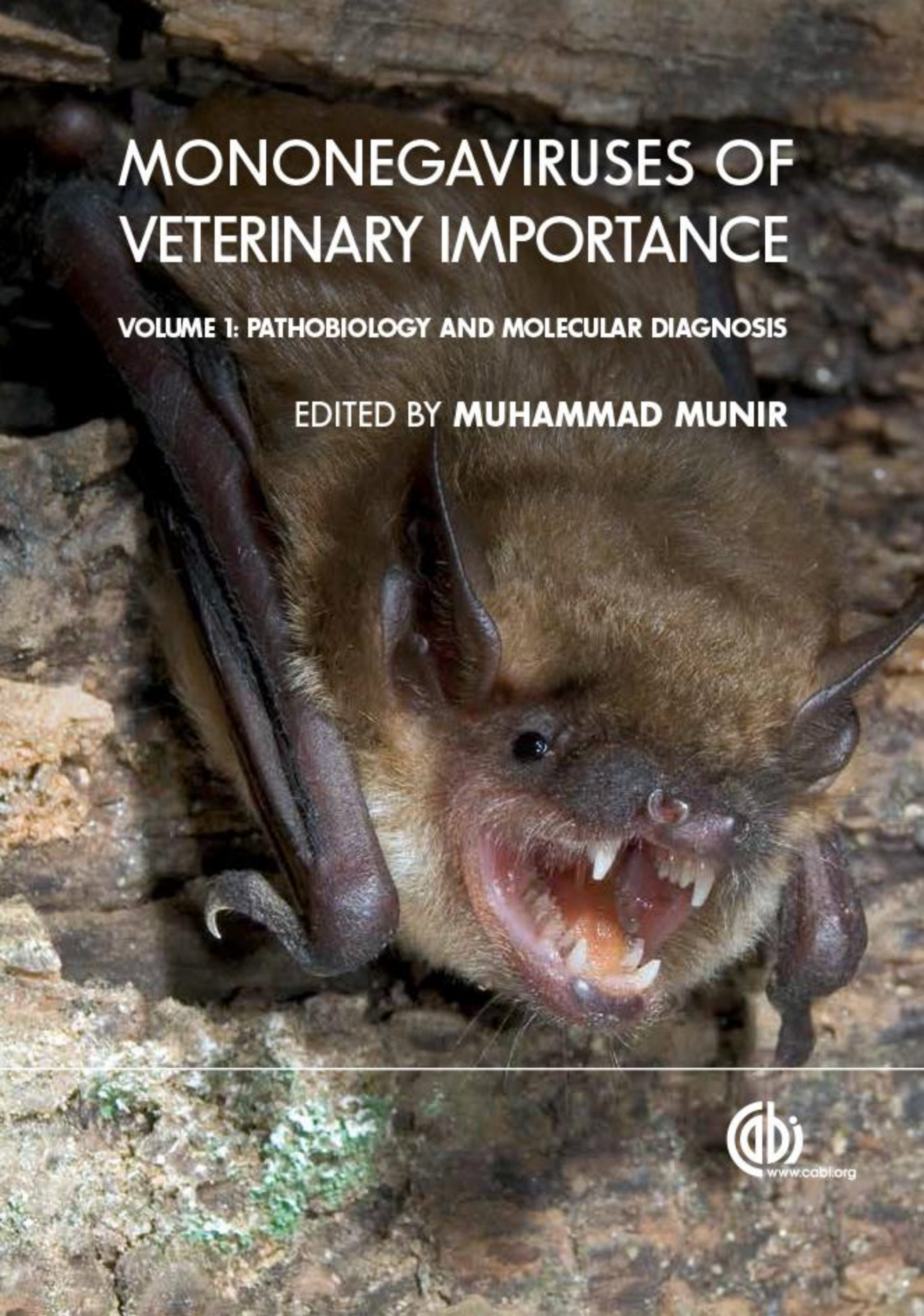

MONONEGAVIRUSES OF VETERINARY IMPORTANCE

VOLUME 1: PATHOBIOLOGY AND MOLECULAR DIAGNOSIS

EDITED BY **MUHAMMAD MUNIR**

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Mononegaviruses of Veterinary Importance

Volume I: Pathobiology and Molecular Diagnosis

Dedication

Sándor Belák obtained his veterinary surgeon degree from University of Veterinary Sciences in Budapest in 1969 and worked at the headquarters of the Food and Agricultural Organization (FAO) in Rome and at the Foot-and-Mouth-Disease Vaccine and Diagnostic Institute in Ankara (Turkey). He returned to the University of Veterinary Sciences in Budapest, where he obtained his PhD in 1978. After working for a year at the National Animal Disease Centre in Ames (Iowa, USA), he moved to Sweden in 1985. He excelled as an innovative scientist in his own founded (Joint Research and Development Division of the Departments of Virology (National Veterinary Institute, SVA) and the Swedish University of Agricultural Sciences (SLU)) division. He has earned the *Centenary Award* of the British Veterinary Association (1989), the *George Fleming Literary Prize* (2002), the *Thuréus prize* of the Royal Society of Sciences, Sweden (2004) and the *Köves prize* of the Hungarian Veterinary Association/Ceva (2011). In 2012, he was decorated with the *Gold Medal* of the Royal Swedish Academy of Agriculture and Forestry, KSLA "for outstanding research work in veterinary virology". Carl XVI Gustaf, the King of Sweden, delivered the award at the occasion of the 199th anniversary meeting of the Royal Academy in Stockholm on 28 January 2012.

Beside his excellence in academia, he is a great social worker, a poet, a connoisseur of art, and a polyglot world traveller. I want to see him never boring, always full of ideas, and always stimulating person, for ever.

Muhammad Munir

Mononegaviruses of Veterinary Importance

Volume I: Pathobiology and Molecular Diagnosis

Edited by

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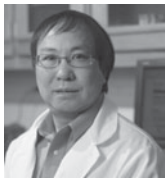

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Preface

The *Mononegavirales* are an order of viruses comprising species that have a linear, non-segmented, non-infectious and negative sense RNA genome. The order includes four families, Bornaviridae (e.g. Borna disease virus), Rhabdoviridae (e.g. Rabies virus), Filoviridae (e.g. Ebola virus) and Paramyxoviridae (e.g. Newcastle disease virus), beside a recently proposed fifth family Nyamiviridae (e.g. Nyamanini virus).

Mononegaviruses (physical members of order *Mononegavirales*) have a history that dates back several tens of millions of years. It has been investigated that genes or gene fragments (fossils) of mononegaviruses are integrated into the mammalian genome. For instance, the genes of Borna disease virus was detected in the genomes of bats, fish, primates, rodents, ruminants, and elephants whereas Filovirus gene "fossils" have been detected in the genomes of bats, rodents, shrews, and marsupials. Beside this old history, several new members of the order such as novel Paramyxoviruses are being reported.

Each family of order *Mononegavirales* contains pathogens that can infect animals or fish. Some mononegaviruses have established themselves in a wide range of host species including mammals, birds, reptile and fish while others are emerging pathogens such as Borna disease virus, Hendra virus and Nipah virus. Moreover, some animal pathogens of this order, such as Ebola virus, Marburg virus, Hendra virus and Nipah virus, carry zoonotic potential that is imparting enormous consequences in term of production losses and health standards. Members such as Sendai virus and Newcastle disease virus have appeared as state-of-the-art models to understand the biology and pathogenesis of veterinary mononegaviruses. Despite of virtually similar genome organization throughout order *Mononegavirales*, these viruses cause diseases of different levels in host-specific manners.

The purpose of this book is to provide timely and comprehensive review on all the mononegaviruses of veterinary importance. The book is divided into two sections. Section I deals 13 mononegaviruses of livestock, horses, dogs and cats whereas Section II comprises of 9 other mononegaviruses of rodents, primates, fish and sea mammals. A full chapter has been dedicated to each virus, which provides up-to-date literature on historical distribution, genome structure, viral proteins, reverse genetics, immunity, viral pathogenesis, clinical and molecular diagnosis, and future challenges. Every chapter is written by renowned scientists who have made seminal contributions in their respective mononegavirus fields of expertise. Each chapter was attempted to make as stand-alone document, making it a valuable reference source for virologists, field

veterinarians, infection and molecular biologists, immunologists, scientists in related fields and veterinary school libraries.

Last but not least, I would like to thank all the authors for their contributions, timely responses and full devotion for completion of this project. I hope that this book will provide a resource to advocate best possible presentation for readers and a networking platform for the contributors.

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1 Bornaviruses

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1.1 Introduction

By the 18th century, a neurological disorder had already been described in a German textbook of equine diseases, and been seen to cause clinical signs in horses in Central Europe (reviewed in Dürrwald and Ludwig, 1997). After an outbreak in the 1890s in the city of Borna, outside Leipzig in the Kingdom of Saxony, the disease was named 'Bornasche Krankheit' or Borna disease (BD). As a result of this outbreak, the Ministry of Home Affairs decided to characterize its clinical signs, pathology and aetiology, with the aim of eventually controlling the disease (Schmidt, 1912). The pathology of BD and the clinical signs were thoroughly investigated in diseased horses by the pathologists Joest and Degen (Joest and Degen, 1911), and the equine clinician Schmidt in the early 20th century (Schmidt, 1912), respectively. Several aetiological causes were proposed: bacteria, bacterial toxins and finally a virus (already suggested by Joest and Degen in 1911). The final proof of a viral cause of BD was established by Zwick and his co-workers (Zwick and Seifried, 1925; Zwick *et al.*, 1928). This was achieved through a series of experiments, including filtration of brain homogenates from BD horses and experimental transmission to rabbits, which concurrently developed the disease.

The physical properties of the virus, designated Borna disease virus (BDV), were recorded in the 1920s and 1930s, and the size of the virus was determined to be around 85–125 nm (Zwick, 1939). The first electron micrographs showed spherical virus particles (Fig. 1.1; Ludwig and Becht, 1977), and the viral genome was suggested to be of RNA nature (Danner, 1977; Ludwig and Becht, 1977); however, the first full-genome sequences and genome characterization were only reported 15 years later (Briese *et al.*, 1994; Cubitt *et al.*, 1994a).

Initially, BDV was thought to be endemic only in parts of Central Europe, where it occasionally caused neurological disease in horses and sheep (Ludwig and Bode, 2000), but the findings of antibodies in humans increased the interest in, and thereby the information about, BDV (Rott *et al.*, 1985; Ludwig and Bode, 2000). The natural host range is now considered to be broad, and BDV has been found to cause neurological disease in not only horses and sheep, but also in cats (Lundgren *et al.*, 1995b; Kamhieh and Flower, 2006; Wensman *et al.*, 2008), dogs (Weissenböck *et al.*, 1998; Okamoto *et al.*, 2002b), cattle (Bode *et al.*, 1994; Caplazi *et al.*, 1994; Okamoto *et al.*, 2002a), goats (Caplazi *et al.*, 1999), rabbits (Metzler *et al.*, 1978) and different ungulates. BDV has also been detected throughout the world on

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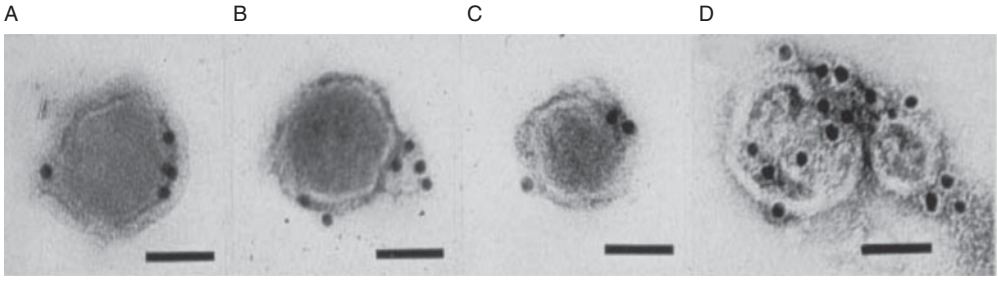

Fig. 1.1. Viral particles of BDV shown by transmission electron microscope. Whole BDV particles from a CsCl fraction of 1.22 g/ml, incubated with a neutralizing rat serum and then stained with anti-rat IgG coupled to 10 nm gold particles (uranyl acetate negative stain). Panel D: Most probably a nucleocapsid is stained besides the virion. Bar = 50 nm. (Photo kindly provided by Hanns Ludwig, Berlin, Germany.)

all continents except Africa (Ludwig and Bode, 2000). Recently, an avian bornavirus (ABV) has been found to be the cause of proventricular dilatation disorder (PDD), which is causing severe losses of psittacine birds in several countries globally (Honkavuori *et al.*, 2008; Kistler *et al.*, 2008; Gray *et al.*, 2010; Payne *et al.*, 2011b).

1.2 Virus Biology and Genome Organization

The genome of BDV and ABV is organized in a similar manner to other members of the order of *Mononegavirales* (Jordan and Lipkin, 2001; Pringle, 2005; Honkavuori *et al.*, 2008; Kistler *et al.*, 2008). Based on its unique use of the nucleus as the replication site (Briese *et al.*, 1992), these viruses belong to the family *Bornaviridae*. The viral genome consists of around 8900 nucleotides with three transcription units, encoding six ORFs (Jordan and Lipkin, 2001), and utilizes the cellular splicing machinery to effectively use its comparatively short genome (Cubitt *et al.*, 1994b; Schneider *et al.*, 1994). The first transcription unit encodes one ORF, resulting in the nucleoprotein (N), while the second transcription unit consists of two overlapping ORFs for the phosphoprotein (P) and the non-structural p10 protein (X) (Jordan and Lipkin, 2001; Tomonaga *et al.*, 2002). The third transcription unit uses alternative splicing, and also has different transcription initiation and termination signals, which enable polymerase read-through during transcription, resulting in the expression of the matrix protein (M), the glycoprotein (G) and the large protein or RNA-dependent RNA-polymerase (L) (Jordan and Lipkin, 2001; Tomonaga *et al.*, 2002).

The BDV genome is highly conserved, where most of the isolated strains have sequence similarities of at least 95% (Lipkin and Briese, 2006). So far only two genotypes have been observed, where the more divergent genotype (strain No/98), isolated from an Austrian horse, differs about 15% in comparison to the other genotype (Nowotny *et al.*, 2000). The ABV genome seems to be more variable, with greater in-between genetic variation compared with mammalian BDV strains, and comprises at least five genotypes infecting psittacine birds (Staeheli *et al.*, 2010). There are also other distinct lineages of ABV infecting non-psittacine birds (Staeheli *et al.*, 2010; Payne *et al.*, 2011a).

The proteins encoded by the viral genome have similar functions as in other members of the same order. The N protein locates primarily inside the nucleus of infected cells and is the most common viral protein (de la Torre, 2002). The N protein constitutes the backbone of the nucleocapsid or ribonucleoprotein (RNP) together with the genomic RNA (Hock *et al.*, 2010), and also interacts with the P protein (Berg *et al.*, 1998b). The N and P proteins are important for the intracellular transport of

RNPs to and from the nucleus (Kobayashi *et al.*, 2001). The P protein of BDV is a co-factor of the viral polymerase complex; however, unlike other viruses within the order of *Mononegavirales*, BDV P is a negative regulator of the polymerase activity upon phosphorylation (Schmid *et al.*, 2007). An outer layer covers the RNP and this layer probably protects the genomic RNA and the nucleocapsid proteins. This layer consists of M protein, which interacts with P and binds to single-stranded RNA, likely the genomic RNA (Chase *et al.*, 2007; Neumann *et al.*, 2009). The G protein of BDV is a precursor molecule, cleaved by cellular proteases into two biologically active proteins, GP-1 and GP-2 (Richt *et al.*, 1998), responsible for receptor attachment to the host cell and endosomal release of the RNPs, respectively (Gonzalez-Dunia *et al.*, 1998; Perez *et al.*, 2001; Clemente and de la Torre, 2009). The RNA-dependent RNA-polymerase complex consists of the large protein (L), N and P (Schneider, 2005). The activity of this complex is regulated through phosphorylation of the L and P proteins (Walker *et al.*, 2000; Schmid *et al.*, 2007). The latter is due to the interaction between P and X (Poenisch *et al.*, 2004). The X protein has important functions for promoting a persistent infection; it can inhibit apoptosis (Poenisch *et al.*, 2009) and interfere with the type I interferon (IFN) signaling pathway (Wensman *et al.*, 2013). The molecular mechanisms behind these important functions of X protein are still unknown.

Certain viruses, such as retroviruses and herpesviruses, are known to insert parts of their DNA into the host genome. This mechanism can be a vital part of the life cycle of the virus, for viral replication or to avoid the immune system of the host. It can also be beneficial for the host as a way to gain new genetic material needed for its survival (Koonin, 2010). Recently, BDV-like gene elements have been found integrated into mammalian genomes, including the human genome, of which some results in protein expression (Belyi *et al.*, 2010; Horie *et al.*, 2010). These integration events occurred several millions of years ago. To facilitate genomic integration, the viral RNA genome needs to be converted into the complementary DNA. Evidently, such cDNA-conversion can occur in acutely BDV-infected animals, but it is not known whether this leads to integration into the host genome (Horie *et al.*, 2010; Kinnunen *et al.*, 2011). Genetic elements from other *Mononegavirales*, Ebola and Marburg viruses, have also been found integrated into mammalian host genomes (Belyi *et al.*, 2010). Whether these integrations are a way for BDV to avoid the host immune response or beneficial for the host is not known.

1.3 Virus Life Cycle

Bornaviruses are unique among animal RNA-viruses in using the nucleus as the replication site (Briese *et al.*, 1992); it is only the *Orthomyxoviruses* among RNA-viruses that also replicate within the nucleus. Bornavirus infection leads to non-cytolytic persistency, mainly in cells of the nervous system, but most cell lines are susceptible to infection *in vitro* (Ludwig, 2008).

The virus enters the host cell by attaching its GP-1 to a cellular surface receptor of yet unknown identity, followed by a clathrin-mediated endocytosis facilitated by the receptor binding (Gonzalez-Dunia *et al.*, 1998; Perez *et al.*, 2001; Clemente and de la Torre, 2009). Inside the cytoplasm, the encapsulated virus releases from the early endosome as RNP through a pH-dependent fusion of the viral and endosomal membranes, mediated by GP-2 (Gonzalez-Dunia *et al.*, 1998; Clemente and de la Torre, 2009). The RNPs are transported into the nucleus through several nuclear localization signals (NLSs) present in the N P and L proteins, where of the NLSs in BDV, N are considered to be the most important (de la Torre, 2002). Inside the nucleus, the RNPs are tightly connected to the host chromatin, and generate viral factories where replication and transcription occur (Matsumoto *et al.*, 2012). These factories can be seen as intranuclear inclusion bodies, called Joest–Degen bodies after the pathologists that first described them (Joest and Degen, 1911). In dividing cells, infectious RNPs can spread to daughter cells by the binding to the host

chromosomes (Matsumoto *et al.*, 2012), giving the means to a persistent nuclear infection. During replication, the 5′-triphosphate group is exchanged into a monophosphate group by genome trimming (Schneider *et al.*, 2005). This process is a way for BDV and ABV to avoid pathogen recognition by the host (Habjan *et al.*, 2008; Reuter *et al.*, 2010). The site and process of assembly and maturation of viral particles have not yet been fully identified, and it is not known to what extent budding of viral particles occurs in natural infection (de la Torre, 2002). BDV mainly associates to infected cells, and only small amounts are released (Ludwig and Becht, 1977). Inside the central nervous system (CNS), the virus is likely to spread as RNPs by cell-to-cell contact, because no virus particles have been observed within the CNS (Gosztonyi *et al.*, 1993). This observation is also confirmed in BDV-infected cell cultures (Clemente and de la Torre, 2007).

1.4 Pathogenesis

There are still many uncertainties about how BDV and ABV spread between animals. Because of geographical, seasonal and annual differences in BDV-infected animals, it has been hypothesized that natural reservoirs are involved (Staeheli *et al.*, 2000). Some potential reservoirs have also been identified, such as birds (Berg *et al.*, 2001), insectivores (Hilbe *et al.*, 2006) and rodents (Kinnunen *et al.*, 2007, 2011). There are also arguments for direct spread of BDV in horses, because of the high level of seroprevalence found by some researchers (Ludwig, 2008). On the other hand, there seems to be little cat-to-cat spread of BDV, as most often only single cats in multi-cat households show clinical signs of BDV infection (Berg *et al.*, 1998a). The route of transmission of ABV is likely faecal–oral, but airborne transmission cannot be excluded (Hoppes *et al.*, 2010).

Natural infection of BDV leads to progressive neurological signs, starting with more diffuse clinical signs such as disturbances in feed intake (horses and sheep), fever (all animals) and different degrees of somnolence (Schmidt, 1912; Heinig, 1969; Richt *et al.*, 2000; Wensman *et al.*, 2012). The neurological signs comprise gait disturbances, such as circular movement (horses, sheep and cattle) and ataxia (cats), postural deficits and behavioural changes (all animals) (reviewed in Wensman, 2012). Natural BDV infection most often leads to euthanasia, because of the bad prognosis and decreased life quality due to neurological impairment. ABV infection of psittacine birds leads to proventricular dilatation disorder (PDD), characterized by depression, weight loss, passage of undigested feed and gait disturbances (Gregory *et al.*, 1994).

Most likely, BDV first enters cells of the olfactory epithelium and oro-pharyngeal mucosa (Morales *et al.*, 1988; Sauder and Staeheli, 2003), through the binding of GP-1 to a cellular receptor (Gonzalez-Dunia *et al.*, 1998; Perez *et al.*, 2001). This binding leads to viral uptake by endocytosis, followed by release of RNPs from the endosome to the cytoplasm through a pH-dependent fusion mediated by the viral GP-2 (Gonzalez-Dunia *et al.*, 1998; Clemente and de la Torre, 2009). The naked RNP is transported into the nucleus where the transcription and replication take place. From the initial site of replication, i.e. the cells of the olfactory epithelium, BDV is transported as RNPs to the CNS, probably by the use of the cellular macromolecular axonal transport system, and reaches the olfactory bulb within approximately 4–6 days, as shown by experimental intra-nasal infection (Carbone *et al.*, 1987; Gosztonyi and Ludwig, 1995; Gosztonyi, 2008). After reaching the olfactory bulb, BDV spreads to the higher olfactory pathways within the limbic system, and is then distributed to the whole cortical area (Gosztonyi, 2008). The viral distribution inside the CNS is trans-neural (direct transmission from cell-to-cell) most likely by RNPs, and not by membrane-enveloped viral particles (Gosztonyi *et al.*, 1993; Clemente and de la Torre, 2007). This strategy of viral transmission could be one way to overcome the host immune response, but it could also be the result of a specific neuronal defence mechanism to avoid

cytopathic infection, followed by destruction of the important neurons of the CNS (Patterson *et al.*, 2002).

The virus–host interactions responsible for the trans-neural spread of RNPs are not known, but the binding between the glutamate kainate 1 (KA-1) receptor and the viral M protein has been proposed (Gosztonyi and Ludwig, 2001; Gosztonyi, 2008). A few months after infection, BDV spreads to the spinal cord and to cranial and peripheral nerves, including those of the autonomic nervous system (Gosztonyi and Ludwig, 1995; Gosztonyi, 2008). This spread results in blindness, due to neuronal degeneration of infected retinal neurons (Krey *et al.*, 1979; Dietzel *et al.*, 2007), and secretion of infectious membrane-enveloped virus particles, due to infection of visceral organs (Gosztonyi and Ludwig, 1995; Gosztonyi, 2008). In naturally infected animals, infectious BDV has been detected in lacrimal and nasal secretions (Richt *et al.*, 2000), but viral RNA has also been found by RT-PCR in several other different body fluids or excretions, such as peripheral blood, saliva, urine and faeces (Richt *et al.*, 1993; Vahlenkamp *et al.*, 2000, 2002; Wensman *et al.*, 2012). In experimentally infected rats, clinical disease first appears when BDV antigens are expressed in hippocampal neurons, which gives rise to an inflammatory reaction (Carbone *et al.*, 1987). Therefore, the incubation time depends on the route of infection. The incubation time in naturally infected animals is estimated from 3 weeks to 6 months, probably depending on infection route, infection dose and species (Heinig, 1969; Mayr and Danner, 1974; Ludwig and Kao, 1988). Vertical transmission of BDV has been reported in horses (Hagiwara *et al.*, 2000), although this has not been confirmed by other investigators (Richt *et al.*, 2000).

The pathogenesis of ABV infection in psittacine birds has not been fully explored to date, but experimental infections show incubation times from approximately 1 month up to 6 months (Gancz *et al.*, 2009; Piepenbring *et al.*, 2012). There is evidence of a faecal–oral transmission route, but airborne transmission cannot be excluded (Hoppes *et al.*, 2010). Whether the same uptake mechanism as for BDV is true also for ABV is not known. Intracerebral and intravenous inoculation both give clinical signs of PDD, as well as a wide distribution of ABV in different organs, preferably in the CNS, gastrointestinal (GI) system and skin with feathers (Piepenbring *et al.*, 2012). Preliminary data show a possible vertical transmission of ABV from infected parents to the embryo (Lierz *et al.*, 2011).

The histopathological features of both BDV and ABV infection are non-suppurative lymphoplasmacytic or lymphohistiocytic inflammation of the CNS, predominantly seen in the grey matter of the brain stem, basal nuclei and hippocampus (Joest and Degen, 1911; Gosztonyi and Ludwig, 1984; Lundgren, 1992; Hoppes *et al.*, 2010), but in ABV-infected birds other tissues, such as the autonomic ganglia of the GI-tract, also have prominent lesions and antigen expression (Raghav *et al.*, 2010). Inflammatory lesions of intra-abdominal autonomic ganglia and the medulla of the adrenal gland have been shown in BDV-infected cats (Wensman *et al.*, 2012), and similar findings are known in experimentally infected rats (Gosztonyi and Ludwig, 1995). In some mammalian species, pathognomonic, intranuclear inclusion bodies can be found in neurons, called Joest–Degen inclusion bodies (Joest and Degen, 1911; Gosztonyi and Ludwig, 1984), corresponding to the viral factories where replication and transcription occur (Matsumoto *et al.*, 2012).

BDV infection in immune-competent animals leads to induction of a T-cell immune response (Stitz *et al.*, 1995), and thereby BD has been considered to be an immune-mediated disease, where the heavy immune response contributes to the development of clinical signs, as cytokine expression has adverse effects on the normal functions of the CNS. In viral infections of the CNS, non-cytolytic clearance of virus is essential to spare the non-renewable neurons (Griffin, 2003). Therefore, $CD4^+$ and $CD8^+$ T cells and antibodies are more important for virus clearance than the neurotoxic IFN-α (Griffin, 2003). The mechanisms of virus clearance differ between the different

cells of the CNS. Neurons are mainly protected by antibodies, locally produced by B cells, and IFN-γ, produced by T cells and the neuron itself (Griffin, 2003). For virus clearance of glial cells, T cells in combination with IFN-γ are important. Although virus clearance can be effective, the virus can still persist. The immune response of naturally BDV-infected animals has only been studied in horses and cats, but there seem to be many similarities to the immune response of experimentally infected rats. In BDV-infected animals, mononuclear perivascular cuffs are frequently present in the CNS, and in these cuffs $CD4^+$ T cells are the dominating cell type (Bilzer *et al.*, 1995; Lundgren *et al.*, 1995a; Stitz *et al.*, 2002). Cytotoxic $CD8^+$ T cells are more common in the brain parenchyma, at least in horses (Bilzer *et al.*, 1995; Lundgren *et al.*, 1995a; Stitz *et al.*, 2002). Locally produced antibodies can be found in experimental infection (Deschl *et al.*, 1990), and plasma cells are seen next to infected neurons in naturally infected cats (Lundgren *et al.*, 1995a). In experimentally infected mice, IFN-γ is crucial for $CD8^+$ T cell-mediated BDV clearance (Hausmann *et al.*, 2005), and IFN-γ mRNA is expressed in BDV-infected cats (Wensman *et al.*, 2011). Even though the antiviral immune response of the CNS is active in trying to clear BDV from the infected brain, viral persistence is most often seen; however, there might be species differences as BDV-infected cats express comparably lower amount of BDV antigens than horses (Gosztonyi and Ludwig, 1984; Lundgren *et al.*, 1995a).

There is growing evidence pointing towards more direct virus-induced effects in the development of clinical signs than previously considered, e.g. BDV-infected cats showing mild neurological signs have severe or moderate inflammatory lesions together with higher expression of IFN-γ mRNA than cats with more severe neurological signs (Wensman *et al.*, 2011, 2012). Moreover, in experimental infection of neonatal rats, BDV infection in the absence of inflammatory reaction develops pathological and behavioural alterations (Gosztonyi and Ludwig, 1995), and transgenic mice expressing BDV P in glial cells develop similar neurological signs as BDV-infected animals (Kamitani *et al.*, 2003). Several BDV–host protein–protein interactions have also been reported, resulting in interference of important cellular signaling pathways (Planz *et al.*, 2009). These interactions are summarized, together with the interactions between the viral proteins, in Fig. 1.2. BDV N reduces the cell proliferation rate by binding to the Cdc2-Cyclin B1 complex (Planz *et al.*, 2003), involved in the G_2-M phase transition of the cell division cycle (Castedo *et al.*, 2002). This interference could be a way for BDV to enable a persistent infection. BDV P can act as a decoy substrate of cellular kinases, and thereby competitively interfere with the phosphorylation of endogenous substrates, which leads to decreased type I IFN-expression (when phosphorylated by TBK-1; Unterstab *et al.*, 2005) and reduced neuronal activity, reduced neuronal plasticity and increased viral transmission (upon phosphorylation by protein kinase Cε; Prat *et al.*, 2009; Schmid *et al.*, 2010). BDV P can also interfere with the γ-aminobutyric acid receptor (GABA-R) trafficking system, by interacting with the GABA-R associated protein (GABARAP) (Fig. 1.3; Peng *et al.*, 2008). This interaction leads to a decreased transport of GABA-R to the cellular membrane, a mechanism possibly responsible for some of the behavioural changes seen in BDV infection (Crestani *et al.*, 1999). BDV P can also interact with the nuclear multi-functional high-mobility group box-1 (HMGB1) protein, resulting in reduced neurite outgrowth (Kamitani *et al.*, 2001), reduced p53-mediated transcription (Zhang *et al.*, 2003), and reduced pro-inflammatory response (Kamitani *et al.*, 2001; Wensman, 2012). The binding of BDV P to HMGB1 also stabilizes the BDV RNP interaction with the host chromatin, facilitating the persistent infection of the nucleus (Matsumoto *et al.*, 2012). BDV has evolved another immune evasion strategy by inhibiting the gene expression of inducible nitric oxide synthase (iNOS) induced by IFN-γ through the P (Peng *et al.*, 2007). Other evasion strategies are ascribed to BDV X, which induces apoptosis resistance in BDV-infected

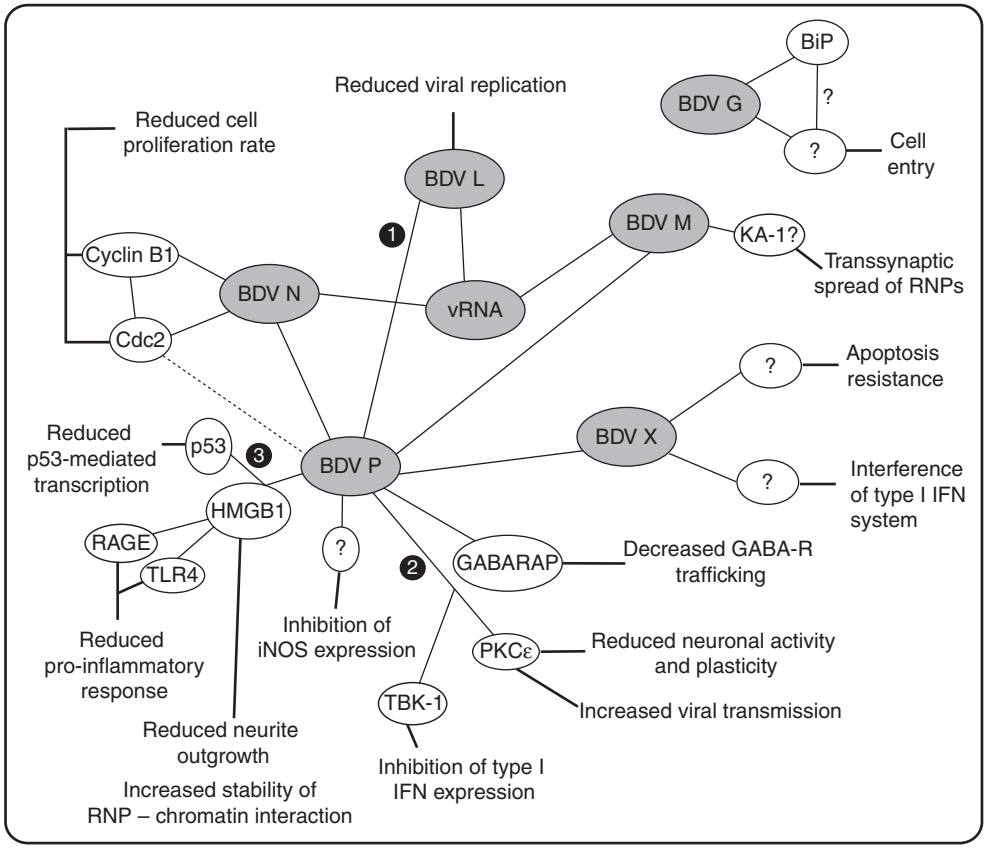

Fig. 1.2. Interactions between the viral components, viral and host cellular proteins, and the biological impact of these interactions. Shaded circles: BDV proteins and viral RNA; empty circles: host cellular proteins. Phosphorylated BDV P (1) downregulates the activity of the viral polymerase (BDV L), (2) acts as decoy substrate for TBK-1 and PKCε phosphorylation and (3) competes with p53 for the same binding site of HMGB1.

cells (Poenisch *et al.*, 2009) and interferes with the type I IFN signaling pathways (Wensman *et al.*, 2013).

1.5 Diagnostics

It is a challenging task to make a clinical diagnosis of Bornavirus infection in a living animal. Even though the clinical signs are similar in different species, they are not specific, and due to the nature of a persistent CNS infection, markers of infection, such as BDV-specific antibodies, antigen or nucleic acids, are difficult to detect. Therefore, the clinical diagnosis of BDV infection is mainly a tentative diagnosis, made by excluding other potential causes of the clinical signs, although presence of clinical signs, viral nucleic acids, viral proteins and/or virus-specific antibodies and pathological changes of the cerebrospinal fluid (CSF) in accordance with BDV infection give a reliable diagnosis (Richt *et al.*, 2000; Wensman *et al.*, 2012). Detection of antibodies or antigen in the CSF is considered to confirm an on-going BDV infection, since these are only detected in diseased animals (Richt *et al.*, 2000). BDV-specific antibodies in serum are found in both healthy and diseased animals, although to different levels of seroprevalence. In regions where BDV infections are considered

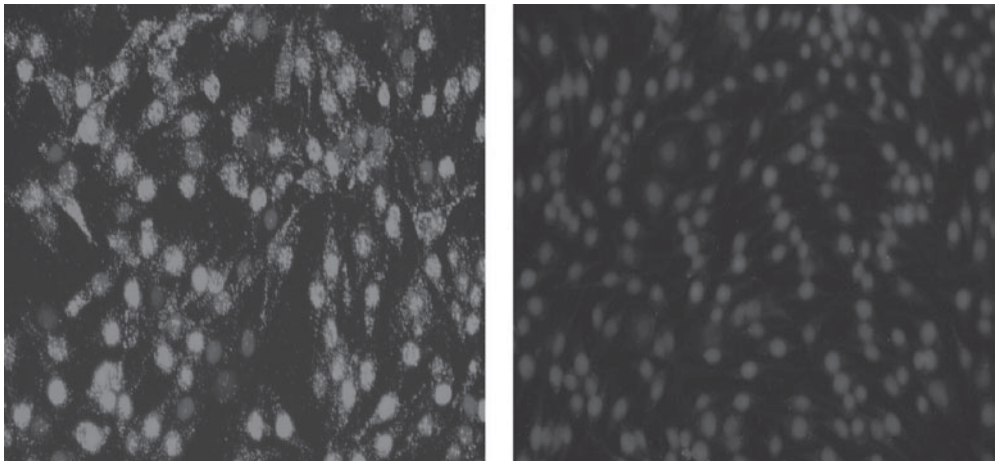

Fig. 1.3. Interactions between BDV P (left) or N (right), and GABARAP in BDV-infected rat astrocytes. To visualize the interactions, *in situ* PLA was applied (Duolink, Olink Biosciences, Uppsala, Sweden). The bright white staining indicates BDV–GABARAP interactions. (Photo: Karl-Johan Leuchowius and Jonas J. Wensman.)

endemic, approximately 20% of the healthy population carry BDV-specific antibodies, whereas the corresponding seroprevalence for BDV-infected animals is around 80% when using indirect immunofluorescence assay (IFA) (Richt *et al.*, 2000; Wensman *et al.*, 2012). An ELISA targeting circulating immune-complexes (antibody–antigen complexes) seems to be more sensitive (Bode *et al.*, 2001), as up to 60% of the horse population in endemic regions are reported seropositive (Ludwig and Bode, 2000; Ludwig, 2008). Viral nucleic acids have been found in peripheral blood and different body secretions, both from subclinical and clinical cases (Richt *et al.*, 1993; Vahlenkamp *et al.*, 2000, 2002; Wensman *et al.*, 2012). Molecular diagnostics, as serology, therefore needs to be interpreted with caution, and always in combination with evaluation of the clinical signs, clinical history and epidemiology. Because of high sequence similarities between different BDV isolates, special precautions are needed to avoid contamination of samples (Wensman *et al.*, 2007, 2011, 2012a).

Today, a confirmed diagnosis of BDV infection is achieved when performing necropsy and histopathological examinations, followed by detection of virus. In some species, especially horses, intra-nuclear Joest–Degen inclusion bodies can sometimes be found, and these are considered pathognomonic for BDV infection (Joest and Degen, 1911; Gosztonyi and Ludwig, 1984). Detection of BDV-antigen and/or -RNA is done by immunohistochemistry (IHC), *in situ* hybridization (ISH) and RT-PCR; however, some species, such as cats, have low viral loads resulting in weaker IHC and ISH signals (Lundgren *et al.*, 1995a), and comparatively high Ct-values in real-time RT-PCR (Wensman *et al.*, 2007, 2011, 2012).

PDD, as a result of ABV infection, can be confirmed by crop biopsy and histopathological examination, which is considered the golden standard, although a negative result does not exclude the diagnosis (Gancz *et al.*, 2010). Diagnostic imaging can be helpful but not specific, showing a dilated proventriculus (Gancz *et al.*, 2010). Serological analysis by IFA and detection of ABV-RNA guide the clinician making the diagnosis, especially in combination, where birds with high viral loads and high titres of antibodies are at high risk of developing PDD (Heffels-Redmann *et al.*, 2012).

1.6 Conclusion

BDV and ABV are unique viruses among the members of *Mononegavirales*, as they are

replicating in the nucleus of animal host cells. They cause persistent infection, mainly in cells of the CNS, but especially in the case of ABV also in other organs. The infection results in progressive neurological disorders of mammals and birds, and most often diseased animals need to be euthanized. To make the clinical diagnosis of BDV and ABV infection, clinical signs, clinical history and epidemiology in combination with markers of virus infection, such as antibodies, antigens or nucleic acids, as well as excluding differential diagnoses, are needed. Histopathological examination, followed by virus detection in tissues, confirms the clinical diagnosis. The pathogenesis is not fully understood, but the clinical signs are most likely the result of the heavy inflammatory reaction, as well as direct effects mediated by virus–host protein–protein interactions, adversely affecting the normal functions of the CNS. The recent findings of BDV-like elements inserted into the genome of mammals millions of years ago, including the human genome, give rise to further questions regarding these intriguing viruses.

References

Belyi, V.A., Levine, A.J. and Skalka, A.M. (2010) Unexpected inheritance: multiple integrations of ancient Bornavirus and Ebolavirus/Marburgvirus sequences in vertebrate genomes. *PLoS Pathogen* 6, e1001030.

Berg, A.L., Reid-Smith, R., Larsson, M. and Bonnett, B. (1998a) Case control study of feline Borna disease in Sweden. *Veterinary Record* 142, 715–717.

Berg, M., Ehrenborg, C., Blomberg, J., Pipkorn, R. and Berg, A.L. (1998b) Two domains of the Borna disease virus p40 protein are required for interaction with the p23 protein. *Journal of General Virology* 79, 2957–2963.

Berg, M., Johansson, M., Montell, H. and Berg, A.L. (2001) Wild birds as a possible natural reservoir of Borna disease virus. *Epidemiology and Infection* 127, 173–178.

Bilzer, T., Planz, O., Lipkin, W.I. and Stitz, L. (1995) Presence of cd4+ and cd8+ t cells and expression of MHC class I and MHC class II antigen in horses with Borna disease virus-induced encephalitis. *Brain Pathology* 5, 223–230.

Bode, L., Dürrwald, R. and Ludwig, H. (1994) Borna virus infections in cattle associated with fatal neurological disease. *Veterinary Record* 135, 283–284.

Bode, L., Reckwald, P., Severus, W.E., Stoyloff, R., Ferszt, R., Dietrich, D.E. and Ludwig, H. (2001) Borna disease virus-specific circulating immune complexes, antigenemia, and free antibodies – the key marker triplet determining infection and prevailing in severe mood disorders. *Molecular Psychiatry* 6, 481–491.

Briese, T., de la Torre, J.C., Lewis, A., Ludwig, H. and Lipkin, W.I. (1992) Borna disease virus, a negative-strand RNA virus, transcribes in the nucleus of infected cells. *Proceedings of the National Academy of Sciences USA* 89, 11486–11489.

Briese, T., Schneemann, A., Lewis, A.J., Park, Y.S., Kim, S., Ludwig, H. and Lipkin, W.I. (1994) Genomic organization of Borna disease virus. *Proceedings of the National Academy of Sciences USA* 91, 4362–4366.

Caplazi, P., Waldvogel, A., Stitz, L., Braun, U. and Ehrensperger, F. (1994) Borna disease in naturally infected cattle. *Journal of Comparative Pathology* 111, 65–72.

Caplazi, P., Melzer, K., Goetzmann, R., Rohner-Cotti, A., Bracher, V., Zlinszky, K. and Ehrensperger, F. (1999) Die 'Bornasche Krankheit' in der Schweiz und im Fürstentum Lichtenstein. [Borna disease in Switzerland and in the principality of Liechtenstein]. *Schweizische Archiv für Tierheilkunde* 141, 521–527.

Carbone, K.M., Duchala, C.S., Griffin, J.W., Kincaid, A.L. and Narayan, O. (1987) Pathogenesis of Borna disease in rats: evidence that intra-axonal spread is the major route for virus dissemination and the determinant for disease incubation. *Journal of Virology* 61, 3431–3440.

Castedo, M., Perfettini, J.L., Roumier, T. and Kroemer, G. (2002) Cyclin-dependent kinase-1: linking apoptosis to cell cycle and mitotic catastrophe. *Cell Death and Differentiation* 9, 1287–1293.

Chase, G., Mayer, D., Hildebrand, A., Frank, R., Hayashi, Y., Tomonaga, K. and Schwemmle, M. (2007) Borna disease virus matrix protein is an integral component of the viral ribonucleoprotein complex that does not interfere with polymerase activity. *Journal of Virology* 81, 743–749.

Clemente, R. and de la Torre, J.C. (2007) Cell-to-cell spread of Borna disease virus proceeds in the absence of the virus primary receptor and furin-mediated processing of the virus surface glycoprotein. *Journal of Virology* 81, 5968–5977.

Clemente, R. and de la Torre, J.C. (2009) Cell entry of Borna disease virus follows a clathrin-mediated endocytosis pathway that requires rab5 and microtubules. *Journal of Virology* 83, 10406–10416.

Crestani, F., Lorez, M., Baer, K., Essrich, C., Benke, D., Laurent, J.P., Belzung, C., Fritschy, J.M., Luscher, B. and Mohler, H. (1999) Decreased GABAA-receptor clustering results in enhanced anxiety and a bias for threat cues. *Nature Neuroscience* 2, 833–839.

Cubitt, B., Oldstone, C. and de la Torre, J.C. (1994a) Sequence and genome organization of Borna disease virus. *Journal of Virology* 68, 1382–1396.

Cubitt, B., Oldstone, C., Valcarcel, J. and de la Torre, J.C. (1994b) RNA splicing contributes to the generation of mature mRNAs of Borna disease virus, a non-segmented negative strand RNA virus. *Virus Research* 34, 69–79.

Danner, K. (1977) Borna disease: patterns of infection. In: ter Meulen, V. and Katz, M. (eds) *Slow Virus Infections of the Central Nervous System*. Springer, New York.

de la Torre, J.C. (2002) Molecular biology of Borna disease virus and persistence. *Frontiers in Bioscience* 7, d569–579.

Deschl, U., Stitz, L., Herzog, S., Frese, K. and Rott, R. (1990) Determination of immune cells and expression of major histocompatibility complex class II antigen in encephalitic lesions of experimental Borna disease. *Acta Neuropathologica* 81, 41–50.

Dietzel, J., Kuhrt, H., Stahl, T., Kacza, J., Seeger, J., Weber, M., Uhlig, A., Reichenbach, A., Grosche, A. and Pannicke, T. (2007) Morphometric analysis of the retina from horses infected with the Borna disease virus. *Veterinary Pathology* 44, 57–63.

Dürrwald, R. and Ludwig, H. (1997) Borna disease virus (BDV), a (zoonotic?) worldwide pathogen. A review of the history of the disease and the virus infection with comprehensive bibliography. *Zentralblatt für Veterinärmedizin B* 44, 147–184.

Gancz, A.Y., Kistler, A.L., Greninger, A.L., Farnoushi, Y., Mechani, S., Perl, S., Berkowitz, A., Perez, N., Clubb, S., DeRisi, J.L., Ganem, D. and Lublin, A. (2009) Experimental induction of proventricular dilatation disease in cockatiels (*Nymphicus hollandicus*) inoculated with brain homogenates containing avian Bornavirus 4. *Virology Journal* 6, 100.

Gancz, A.Y., Clubb, S. and Shivaprasad, H.L. (2010) Advanced diagnostic approaches and current management of proventricular dilatation disease. *Veterinary Clinics of North America. Exotic Animal Practice* 13, 471–494.

Gonzalez-Dunia, D., Cubitt, B. and de la Torre, J.C. (1998) Mechanism of Borna disease virus entry into cells. *Journal of Virology* 72, 783–788.

Gosztonyi, G. (2008) Natural and experimental Borna disease virus infections – neuropathology and pathogenetic considerations. *APMIS Supplementum* 116, 53–57.

Gosztonyi, G. and Ludwig, H. (1984) Borna disease of horses. An immunohistological and virological study of naturally infected animals. *Acta Neuropathologica* 64, 213–221.

Gosztonyi, G. and Ludwig, H. (1995) Borna disease – neuropathology and pathogenesis. *Current Topics in Microbiology and Immunology* 190, 39–73.

Gosztonyi, G. and Ludwig, H. (2001) Interactions of viral proteins with neurotransmitter receptors may protect or destroy neurons. *Current Topics in Microbiology and Immunology* 253, 121–144.

Gosztonyi, G., Dietzschold, B., Kao, M., Rupprecht, C.E., Ludwig, H. and Koprowski, H. (1993) Rabies and Borna disease. A comparative pathogenetic study of two neurovirulent agents. *Laboratory Investigation* 68, 285–295.

Gray, P., Hoppes, S., Suchodolski, P., Mirhosseini, N., Payne, S., Villanueva, I., Shivaprasad, H.L., Honkavuori, K.S., Lipkin, W.I., Briese, T., Reddy, S.M. and Tizard, I. (2010) Use of avian Bornavirus isolates to induce proventricular dilatation disease in conures. *Emerging Infectious Diseases* 16, 473–479.

Gregory, C.R., Latimer, K.S., Niagro, F.D., Ritchie, B.W., Campagnoli, R.P., Norton, T.M., McManamon, R. and Greenacre, C.B. (1994) A review of proventricular dilatation syndrome. *Journal of the Association of Avian Veterinarians* 8, 69–75.

Griffin, D.E. (2003) Immune responses to RNA-virus infections of the CNS. *Nature Reviews in Immunology* 3, 493–502.

Habjan, M., Andersson, I., Klingström, J., Schumann, M., Martin, A., Zimmermann, P., Wagner, V., Pichlmair, A., Schneider, U., Muhlberger, E., Mirazimi, A. and Weber, F. (2008) Processing of genome 5′ termini as a strategy of negative-strand RNA viruses to avoid RIG-I-dependent interferon induction. *PloS One* 3, e2032.

Hagiwara, K., Kamitani, W., Takamura, S., Taniyama, H., Nakaya, T., Tanaka, H., Kirisawa, R., Iwai, H. and Ikuta, K. (2000) Detection of Borna disease virus in a pregnant mare and her fetus. *Veterinary Microbiology* 72, 207–216.

Hausmann, J., Pagenstecher, A., Baur, K., Richter, K., Rziha, H.J. and Staeheli, P. (2005) Cd8 T cells require gamma interferon to clear Borna disease virus from the brain and prevent immune system-mediated neuronal damage. *Journal of Virology* 79, 13509–13518.

Heffels-Redmann, U., Enderlein, D., Herzog, S., Piepenbring, A., Burkle, M., Neumann, D., Herden, C. and Lierz, M. (2012) Follow-up investigations on different courses of natural avian Bornavirus infections in psittacines. *Avian Diseases* 56, 153–159.

Heinig, A. (1969) Die Bornasche Krankheit der Pferde und Schafe. In: Röhrer, H. (ed.) *Handbuch der Virusinfektionen bei Tieren*. Fischer, Jena, Germany.

Hilbe, M., Herrsche, R., Kolodziejek, J., Nowotny, N., Zlinszky, K. and Ehrensperger, F. (2006) Shrews as reservoir hosts of Borna disease virus. *Emerging Infectious Diseases* 12, 675–677.

Hock, M., Kraus, I., Schoehn, G., Jamin, M., Andrei-Selmer, C., Garten, W. and Weissenhorn, W. (2010) RNA induced polymerization of the Borna disease virus nucleoprotein. *Virology* 397, 64–72.

Honkavuori, K.S., Shivaprasad, H.L., Williams, B.L., Quan, P.L., Hornig, M., Street, C., Palacios, G., Hutchison, S.K., Franca, M., Egholm, M., Briese, T. and Lipkin, W.I. (2008) Novel Borna virus in psittacine birds with proventricular dilatation disease. *Emerging Infectious Diseases* 14, 1883–1886.

Hoppes, S., Gray, P.L., Payne, S., Shivaprasad, H.L. and Tizard, I. (2010) The isolation, pathogenesis, diagnosis, transmission, and control of avian Bornavirus and proventricular dilatation disease. *The Veterinary Clinics of North America. Exotic Animal Practice* 13, 495–508.

Horie, M., Honda, T., Suzuki, Y., Kobayashi, Y., Daito, T., Oshida, T., Ikuta, K., Jern, P., Gojobori, T., Coffin, J.M. and Tomonaga, K. (2010) Endogenous non-retroviral RNA virus elements in mammalian genomes. *Nature* 463, 84–87.

Joest, E. and Degen, K. (1911) Untersuchungen über die pathologische Histologie, Pathogenese und postmortale Diagnose der seuchenhaften Gehirn-rückenmarksentzündung (Bornaschen Krankheit) des Pferdes. *Zeitschrift für Infektionskrankheiten der Haustiere* 9, 1–98.

Jordan, I. and Lipkin, W.I. (2001) Borna disease virus. *Reviews in Medical Virology* 11, 37–57.

Kamhieh, S. and Flower, R.L. (2006) Borna disease virus (BDV) infection in cats. A concise review based on current knowledge. *Veterinary Quarterly* 28, 66–73.

Kamitani, W., Shoya, Y., Kobayashi, T., Watanabe, M., Lee, B.J., Zhang, G., Tomonaga, K. and Ikuta, K. (2001) Borna disease virus phosphoprotein binds a neurite outgrowth factor, amphoterin/HMG-1. *Journal of Virology* 75, 8742–8751.

Kamitani, W., Ono, E., Yoshino, S., Kobayashi, T., Taharaguchi, S., Lee, B.J., Yamashita, M., Okamoto, M., Taniyama, H., Tomonaga, K. and Ikuta, K. (2003) Glial expression of Borna disease virus phosphoprotein induces behavioral and neurological abnormalities in transgenic mice. *Proceedings of the National Academy of Sciences USA* 100, 8969–8974.

Kinnunen, P.M., Billich, C., Ek-Kommonen, C., Henttonen, H., Kallio, E.R., Niemimaa, J., Palva, A., Staeheli, P., Vaheri, A. and Vapalahti, O. (2007) Serological evidence for Borna disease virus infection in humans, wild rodents and other vertebrates in Finland. *Journal of Clinical Virology* 38, 64–69.

Kinnunen, P.M., Inkeroinen, H., Ilander, M., Kallio, E.R., Heikkila, H.P., Koskela, E., Mappes, T., Palva, A., Vaheri, A., Kipar, A. and Vapalahti, O. (2011) Intracerebral Borna disease virus infection of bank voles leading to peripheral spread and reverse transcription of viral RNA. *PLoS One* 6, e23622.

Kistler, A.L., Gancz, A., Clubb, S., Skewes-Cox, P., Fischer, K., Sorber, K., Chiu, C.Y., Lublin, A., Mechani, S., Farnoushi, Y., Greninger, A., Wen, C.C., Karlene, S.B., Ganem, D. and DeRisi, J.L. (2008) Recovery of divergent avian Bornaviruses from cases of proventricular dilatation disease: identification of a candidate etiologic agent. *Virology Journal* 5, 88.

Kobayashi, T., Kamitani, W., Zhang, G., Watanabe, M., Tomonaga, K. and Ikuta, K. (2001) Borna disease virus nucleoprotein requires both nuclear localization and export activities for viral nucleocytoplasmic shuttling. *Journal of Virology* 75, 3404–3412.

Koonin, E.V. (2010) Taming of the shrewd: novel eukaryotic genes from RNA viruses. *BMC Biology* 8, 2.

Krey, H., Ludwig, H. and Rott, R. (1979) Spread of infectious virus along the optic nerve into the retina in Borna disease virus-infected rabbits. *Archives of Virology* 61, 283–288.

Lierz, M., Piepenbring, A., Herden, C., Oberhauser, K., Heffels-Redmann, U. and Enderlein, D. (2011) Vertical transmission of avian Bornavirus in psittacines. *Emerging Infectious Diseases* 17, 2390–2391.

Lipkin, W.I. and Briese, T. (2006) Bornaviridae. In: Knipe, D., Howley, P., Griffin, D.E., Lamb, R.A., Martin, M., Roizman, B. and Straus, S. (eds) *Fields Virology*, 5th edn. Lippincott, Williams & Wilkins, Philadelphia, Pennsylvania.

Ludwig, H. (2008) The biology of Bornavirus. *APMIS Supplementum* 116, 14–20.

Ludwig, H. and Becht, H. (1977) Borna, the disease – a summary of our present knowledge. In: ter Meulen, V. and Katz, M. (eds) *Slow Virus Infections of the Central Nervous System*. Springer, New York.

Ludwig, H. and Bode, L. (2000) Borna disease virus: new aspects on infection, disease, diagnosis and epidemiology. *Revue Scientifique et Technique* 19, 259–288.

Ludwig, H. and Kao, M. (1988) Borna disease in sheep. In: Horzinek, M. (ed.) *Virus Infections of Ruminants*. Elsevier, Amsterdam, The Netherlands.

Lundgren, A.L. (1992) Feline non-suppurative meningoencephalomyelitis. A clinical and pathological study. *Journal of Comparative Pathology* 107, 411–425.

Lundgren, A.L., Lindberg, R., Ludwig, H. and Gosztonyi, G. (1995a) Immunoreactivity of the central nervous system in cats with a Borna disease-like meningoencephalomyelitis (staggering disease). *Acta Neuropathologica* 90, 184–193.

Lundgren, A.L., Zimmermann, W., Bode, L., Czech, G., Gosztonyi, G., Lindberg, R. and Ludwig, H. (1995b) Staggering disease in cats: isolation and characterization of the feline Borna disease virus. *Journal of General Virology* 76, 2215–2222.

Matsumoto, Y., Hayashi, Y., Omori, H., Honda, T., Daito, T., Horie, M., Ikuta, K., Fujino, K., Nakamura, S., Schneider, U., Chase, G., Yoshimori, T., Schwemmle, M. and Tomonaga, K. (2012) Bornavirus closely associates and segregates with host chromosomes to ensure persistent intranuclear infection. *Cell Host and Microbe* 11, 492–503.

Mayr, A. and Danner, K. (1974) Persistent infections caused by Borna virus. *Infection* 2, 64–69.

Metzler, A., Ehrensperger, F. and Wyler, R. (1978) Natürliche Bornavirusinfektion beim Kaninchen. [Natural Borna virus infection in rabbits]. *Zentralblatt für Veterinärmedizin B* 25, 161–164.

Morales, J.A., Herzog, S., Kompter, C., Frese, K. and Rott, R. (1988) Axonal transport of Borna disease virus along olfactory pathways in spontaneously and experimentally infected rats. *Medical Microbiology and Immunology* 177, 51–68.

Neumann, P., Lieber, D., Meyer, S., Dautel, P., Kerth, A., Kraus, I., Garten, W. and Stubbs, M.T. (2009) Crystal structure of the Borna disease virus matrix protein (BDV-M) reveals ssRNA binding properties. *Proceedings of the National Academy of Sciences USA* 106, 3710–3715.

Nowotny, N., Kolodziejek, J., Jehle, C.O., Suchy, A., Staeheli, P. and Schwemmle, M. (2000) Isolation and characterization of a new subtype of Borna disease virus. *Journal of Virology* 74, 5655–5658.

Okamoto, M., Furuoka, H., Hagiwara, K., Kamitani, W., Kirisawa, R., Ikuta, K. and Taniyama, H. (2002a) Borna disease in a heifer in Japan. *Veterinary Record* 150, 16–18.

Okamoto, M., Kagawa, Y., Kamitani, W., Hagiwara, K., Kirisawa, R., Iwai, H., Ikuta, K. and Taniyama, H. (2002b) Borna disease in a dog in Japan. *Journal of Comparative Pathology* 126, 312–317.

Patterson, C.E., Daley, J.K. and Rall, G.F. (2002) Neuronal survival strategies in the face of RNA viral infection. *Journal of Infectious Diseases* 186 (Suppl 2), S215–219.

Payne, S., Covaleda, L., Jianhua, G., Swafford, S., Baroch, J., Ferro, P.J., Lupiani, B., Heatley, J. and Tizard, I. (2011a) Detection and characterization of a distinct Bornavirus lineage from healthy Canada geese (*Branta canadensis*). *Journal of Virology* 85, 12053–12056.

Payne, S., Shivaprasad, H.L., Mirhosseini, N., Gray, P., Hoppes, S., Weissenböck, H. and Tizard, I. (2011b) Unusual and severe lesions of proventricular dilatation disease in cockatiels (*Nymphicus hollandicus*) acting as healthy carriers of avian Bornavirus (ABV) and subsequently infected with a virulent strain of ABV. *Avian Pathology* 40, 15–22.

Peng, G., Zhang, F., Zhang, Q., Wu, K., Zhu, F. and Wu, J. (2007) Borna disease virus P protein inhibits nitric oxide synthase gene expression in astrocytes. *Virology* 366, 446–452.

Peng, G., Yan, Y., Zhu, C., Wang, S., Yan, X., Lu, L., Li, W., Hu, J., Wei, W., Mu, Y., Chen, Y., Feng, Y., Gong, R., Wu, K., Zhang, F., Zhang, X., Zhu, Y. and Wu, J. (2008) Borna disease virus P protein affects neural transmission through interactions with gamma-aminobutyric acid receptor-associated protein. *Journal of Virology* 82, 12487–12497.

Perez, M., Watanabe, M., Whitt, M.A. and de la Torre, J.C. (2001) N-terminal domain of Borna disease virus G (p56) protein is sufficient for virus receptor recognition and cell entry. *Journal of Virology* 75, 7078–7085.

Piepenbring, A.K., Enderlein, D., Herzog, S., Kaleta, E.F., Heffels-Redmann, U., Ressmeyer, S., Herden, C. and Lierz, M. (2012) Pathogenesis of avian Bornavirus in experimentally infected cockatiels. *Emerging Infectious Diseases* 18, 234–241.

Planz, O., Pleschka, S., Oesterle, K., Berberich-Siebelt, F., Ehrhardt, C., Stitz, L. and Ludwig, S. (2003) Borna disease virus nucleoprotein interacts with the Cdc2-Cyclin B1 complex. *Journal of Virology* 77, 11186–11192.

Planz, O., Pleschka, S. and Wolff, T. (2009) Borna disease virus: a unique pathogen and its interaction with intracellular signalling pathways. *Cell Microbiology* 11, 872–879.

Poenisch, M., Unterstab, G., Wolff, T., Staeheli, P. and Schneider, U. (2004) The X protein of Borna disease virus regulates viral polymerase activity through interaction with the P protein. *Journal of General Virology* 85, 1895–1898.

Poenisch, M., Burger, N., Staeheli, P., Bauer, G. and Schneider, U. (2009) Protein X of Borna disease virus inhibits apoptosis and promotes viral persistence in the central nervous systems of newborn-infected rats. *Journal of Virology* 83, 4297–4307.

Prat, C.M., Schmid, S., Farrugia, F., Cenac, N., Le Masson, G., Schwemmle, M. and Gonzalez-Dunia, D. (2009) Mutation of the protein kinase C site in Borna disease virus phosphoprotein abrogates viral interference with neuronal signaling and restores normal synaptic activity. *PLoS Pathogen* 5, e1000425.

Pringle, C.R. (2005) Mononegavirales. In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U. and Ball, L.A. (eds) *Virus Taxonomy*. Elsevier Academic Press, London.

Raghav, R., Taylor, M., Delay, J., Ojkic, D., Pearl, D.L., Kistler, A.L., Derisi, J.L., Ganem, D. and Smith, D.A. (2010) Avian Bornavirus is present in many tissues of psittacine birds with histopathologic evidence of proventricular dilatation disease. *Journal of Veterinary Diagnostic Investigation* 22, 495–508.

Reuter, A., Ackermann, A., Kothlow, S., Rinder, M., Kaspers, B. and Staeheli, P. (2010) Avian Bornaviruses escape recognition by the innate immune system. *Viruses* 2, 927–938.

Richt, J.A., Herzog, S., Haberzettl, K. and Rott, R. (1993) Demonstration of Borna disease virus-specific RNA in secretions of naturally infected horses by the polymerase chain reaction. *Medical Microbiology and Immunology* 182, 293–304.

Richt, J.A., Furbringer, T., Koch, A., Pfeuffer, I., Herden, C., Bause-Niedrig, I. and Garten, W. (1998) Processing of the Borna disease virus glycoprotein gp94 by the subtilisin-like endoprotease furin. *Journal of Virology* 72, 4528–4533.

Richt, J.A., Grabner, A. and Herzog, S. (2000) Borna disease in horses. *Veterinary Clinics of North America. Equine Practice* 16, 579–595.

Rott, R., Herzog, S., Fleischer, B., Winokur, A., Amsterdam, J., Dyson, W. and Koprowski, H. (1985) Detection of serum antibodies to Borna disease virus in patients with psychiatric disorders. *Science* 228, 755–756.

Sauder, C. and Staeheli, P. (2003) Rat model of Borna disease virus transmission: epidemiological implications. *Journal of Virology* 77, 12886–12890.

Schmid, S., Mayer, D., Schneider, U. and Schwemmle, M. (2007) Functional characterization of the major and minor phosphorylation sites of the P protein of Borna disease virus. *Journal of Virology* 81, 5497–5507.

Schmid, S., Metz, P., Prat, C.M., Gonzalez-Dunia, D. and Schwemmle, M. (2010) Protein kinase C-dependent phosphorylation of Borna disease virus P protein is required for efficient viral spread. *Archives of Virology* 155, 789–793.

Schmidt, J. (1912) Untersuchungen über das klinische Verhalten der seuchenhaften Gehirnrückenmarksentzündung (Bornaschen Krankheit) des Pferdes nebst angaben über diesbezügliche therapeutische Versuche. *Berliner Tierärztlische Wochenschrift* 28, 581–586, 597–603.

Schneider, P.A., Schneemann, A. and Lipkin, W.I. (1994) RNA splicing in Borna disease virus, a nonsegmented, negative-strand RNA virus. *Journal of Virology* 68, 5007–5012.

Schneider, U. (2005) Novel insights into the regulation of the viral polymerase complex of neurotropic Borna disease virus. *Virus Research* 111, 148–160.

Schneider, U., Schwemmle, M. and Staeheli, P. (2005) Genome trimming: a unique strategy for replication control employed by Borna disease virus. *Proceedings of the National Academy of Sciences USA* 102, 3441–3446.

Staeheli, P., Sauder, C., Hausmann, J., Ehrensperger, F. and Schwemmle, M. (2000) Epidemiology of Borna disease virus. *Journal of General Virology* 81, 2123–2135.

Staeheli, P., Rinder, M. and Kaspers, B. (2010) Avian Bornavirus associated with fatal disease in psittacine birds. *Journal of Virology* 84, 6269–6275.

Stitz, L., Dietzschold, B. and Carbone, K.M. (1995) Immunopathogenesis of Borna disease. *Current Topics in Microbiology and Immunology* 190, 75–92.

Stitz, L., Bilzer, T. and Planz, O. (2002) The immunopathogenesis of Borna disease virus infection. *Frontiers in Bioscience* 7, d541–555.

Tomonaga, K., Kobayashi, T. and Ikuta, K. (2002) Molecular and cellular biology of Borna disease virus infection. *Microbes and Infection* 4, 491–500.

Unterstab, G., Ludwig, S., Anton, A., Planz, O., Dauber, B., Krappmann, D., Heins, G., Ehrhardt, C. and Wolff, T. (2005) Viral targeting of the interferon-{beta}-inducing traf family member-associated

nf-{kappa}b activator (tank)-binding kinase-1. *Proceedings of the National Academy of Sciences USA* 102, 13640–13645.

Vahlenkamp, T.W., Enbergs, H.K. and Müller, H. (2000) Experimental and natural Borna disease virus infections: presence of viral RNA in cells of the peripheral blood. *Veterinary Microbiology* 76, 229–244.

Vahlenkamp, T.W., Konrath, A., Weber, M. and Müller, H. (2002) Persistence of Borna disease virus in naturally infected sheep. *Journal of Virology* 76, 9735–9743.

Walker, M.P., Jordan, I., Briese, T., Fischer, N. and Lipkin, W.I. (2000) Expression and characterization of the Borna disease virus polymerase. *Journal of Virology* 74, 4425–4428.

Weissenböck, H., Nowotny, N., Caplazi, P., Kolodziejek, J. and Ehrensperger, F. (1998) Borna disease in a dog with lethal meningoencephalitis. *Journal of Clinical Microbiology* 36, 2127–2130.

Wensman, J.J. (2012) Borna disease virus and its hosts. *Veterinary Science Tomorrow* 12 (http://www.vetscite.org/publish/articles/000106/print.html) (accessed 22 April 2013).

Wensman, J.J., Thorén, P., Hakhverdyan, M., Belák, S. and Berg, M. (2007) Development of a real-time RT-PCR assay for improved detection of Borna disease virus. *Journal of Virological Methods* 143, 1–10.

Wensman, J.J., Berg, M. and Berg, A.L. (2008) Experiences of Borna disease virus infection in Sweden. *APMIS Supplementum* 116, 46–49.

Wensman, J.J., Ilbäck, C., Hjertström, E., Blomström, A.L., Gustavsson, M.H., Jäderlund, K.H., Ström-Holst, B., Belák, S., Berg, A.L. and Berg, M. (2011) Expression of interferon gamma in the brain of cats with natural Borna disease virus infection. *Veterinary Immunology and Immunopathology* 141, 162–167.

Wensman, J.J., Jäderlund, K.H., Gustavsson, M.H., Hansson-Hamlin, H., Karlstam, E., Lilliehöök, I., Öström, I.L., Belák, S., Berg, M. and Holst, B.S. (2012) Markers of Borna disease virus infection in cats with staggering disease. *Journal of Feline Medicine and Surgery* 14, 573–582.

Wensman, J.J., Munir, M., Thaduri, S., Hörnaeus, K., Rizwan, M., Blomström, A.L., Briese, T., Lipkin, W.I. and Berg, M. (2013) The X proteins of Bornaviruses interfere with type I interferon signaling. *Journal of General Virology* 94, 263–269.

Zhang, G., Kobayashi, T., Kamitani, W., Komoto, S., Yamashita, M., Baba, S., Yanai, H., Ikuta, K. and Tomonaga, K. (2003) Borna disease virus phosphoprotein represses p53-mediated transcriptional activity by interference with HMGB1. *Journal of Virology* 77, 12243–12251.

Zwick, W. (1939) Bornasche Krankheit und Enzephalomyelitis der Tiere. In: Gildenmeister, E., Haagen, E. and Waldmann, O. (eds) *Handbuch der Viruskrankheiten*. Fischer, Jena, Germany.

Zwick, W. and Seifried, O. (1925) Uebertragbarkeit der seuchenhaften Gehirn- und Rückenmarksentzündung des Pferdes (Bornaschen Krankheit) auf kleine Versuchstiere (Kaninchen). *Berliner Tierärztliche Wochenschrift* 41, 129–132.

Zwick, W., Seifried, O. and Witte, J. (1928) Weitere Untersuchungen über die seuchenhafte Gehirnrückenmarksentzündung der Pferde (Bornasche Krankheit). *Zeitschrift für Infektionskrankheiten der Haustiere* 32, 150–179.

2 Avian Paramyxoviruses Serotype 1 to 10

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2.1 Introduction

Almost 85 years after the initial identification of the virulent form of avian paramyxovirus serotype 1 (APMV-1) in Java, Indonesia, Miller and colleagues reported the isolation of a new avian paramyxovirus (APMV) serotype in rock hopper penguins (*Eudyptes chrysocome*) from the Falkland Islands, which was serologically and genetically distinct from all previously known serotypes of avian paramyxoviruses (Miller *et al.*, 2010a). Based on haemagglutination inhibition (HI) and neuraminidase inhibition (NI) assays, APMVs are divided into ten distinct serotypes (APMV 1–10) (Alexander, 2011). The list of serotypes of APMVs is certain to grow; the emergence of H5N1 highly pathogenic avian influenza (HPAI) virus in Southeast Asia as a threat to animal and human health has spurred the development of surveillance programmes to better understand the ecology of avian influenza (AI) viruses in their natural reservoirs around the globe, and in some instances it has provided opportunities for observing other viruses like AMPVs in wild bird populations.

APMV-1, the most studied member of this group, is present worldwide and is known to infect over 200 species of birds naturally or under experimental conditions. The virulent form of APMV-1, synonymous with Newcastle disease virus (NDV), is the economically most important viral disease of poultry causing severe losses not only for the poultry industry in developed counties but more importantly for poultry reared under backyard conditions in many developing countries. The disease has substantial negative effects on income, the amount of dietary protein and food security provided by poultry production by the small flocks in developing countries, which can have extremely serious socio-economic consequences for the well-being of millions of people around the globe. Therefore, NDV is classified by the Office International des Epizooties (OIE-World Organization for Animal Health) as a List A disease (OIE, 2012) for which special action by veterinary authorities will be required and must be reported immediately to the OIE.

Very little is known about the pathogenicity of other APMV serotypes. Besides APMV-1, several other serotypes (APMV-2, -3, -4, -6 and -7) have been associated with disease in domestic poultry (Tumova *et al.*, 1979a; Bankowski *et al.*, 1981; Alexander and Collins, 1982; Redmann *et al.*, 1991; Zhang *et al.*, 2006, 2007), while others, like APMV-5, have been implicated in a severe disease of budgerigars (Nerome *et al.*, 1978).

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2.2 Virus and Disease

Newcastle disease (ND) is a highly contagious disease of poultry, caused by an infection with NDV, which is synonymous with avian paramyxovirus serotype 1, a virus that belongs to the genus *Avulavirus* within the family *Paramyxoviridae*, subfamily *Paramyxovirinae*. Because of the extensive range of susceptible hosts, the virus has been able to establish itself worldwide. Natural or experimental infection has been demonstrated in over 200 species from 27 out of 50 orders within the avian class, indicating that perhaps all birds are susceptible to infection (Kaleta and Baldauf, 1988), but susceptibility to disease differs between hosts. Regarding poultry, chickens and turkeys are regarded as the most sensitive domestic species. In contrast, even though waterfowl are susceptible to infection with APMV-1, geese and ducks are found to be relatively resistant concerning the development of clinical disease (Alexander, 2003). Even with strains that are lethal for chickens, ducks and geese usually show no symptoms or only non-specific signs, such as apathy/lethargy and anorexia, for various periods of time. As a consequence, waterfowl might harbour and shed virulent APMV-1 without showing clinical disease. However, in 1997 and the following years a novel disease entity in domestic geese with unusual epidemiology and pathological changes emerged in some regions of China, where velogenic APMV-1 was isolated (Liu *et al.*, 2003; Wan *et al.*, 2004). The disease was reproduced experimentally and the virus was found to be virulent in chickens. A shift in pathogenicity has also been documented for pigeons. Early reports have already demonstrated that pigeons are susceptible to NDV infection and disease (Doyle, 1927), but only sporadic natural infections were reported (Iyer, 1939; Hanson and Sinha, 1951; Kaschula, 1952; Stewart, 1971; Erickson *et al.*, 1980). However, during the early 1980s, a form of ND-like disease among racing pigeons emerged. It is assumed that the first epicentre of this outbreak was the Middle East (Kaleta *et al.*, 1985), where outbreaks with a high case fatality rate in meat-type pigeons occurred in the late 1970s (Tantawi *et al.*, 1979). Based on mapping with monoclonal antibodies and genetic analysis, this APMV-1 type was subsequently classified as pigeon paramyxovirus-1 (PPMV-1) (Collins *et al.*, 1989; Jestin *et al.*, 1989). By 1983, the virus was present in almost all European countries and subsequently all over the world. PPMV-1 can now be isolated not only from domesticated pigeons but from feral (urban) pigeons, doves and ornamental birds and remains a continuing threat (Alexander, 2011). For example in 2010, 2009 and 2008, 15, 16 and 16 European Union (EU) member states reported the detection of PPMV-1 in pigeons, respectively. This endemic situation of PPMV-1 in pigeons regularly leads to a spill-over to poultry. In the year 2011, both France and Sweden experienced ND outbreaks with PPMV-1 in fattening pigeons and laying hens respectively, affecting four flocks with 16,500 fattening pigeons and 34,500 chickens that had to be euthanized. Loss of the animals, together with official restrictions to poultry holdings within a protection and surveillance zone, epidemiological investigations and negative consequences for trading, resulted in heavy economic costs.

Because of the severe economic consequences of an outbreak of ND, including the negative effect on trade and poultry production in commercial poultry, the disease is reportable to the OIE. However, in view of the wide variation in disease caused by APMV-1 strains, very specific criteria were established for defining an outbreak of ND. The disease is defined as an infection of birds caused by an APMV-1 virus that meets one of the following criteria for virulence: (1) the virus has an intra-cerebral pathogenicity index in day-old chickens (*Gallus gallus domesticus*) of 0.7 or greater, or (2) multiple basic amino acids have been demonstrated in the virus (either directly or deduced) at the C-terminus of the F2 protein and phenylalanine at residue 117, which is the N-terminus of the F1 protein. The term ‘multiple basic amino acids’ refers to at least three arginine or lysine residues between residues 113 and 116.

2.3 Classification

Avian paramyxoviruses are single-stranded, negative-sense, filamentous RNA-viruses, which are classified in the genus *Avulavirus* of subfamily *Paramyxovirinae*, family *Paramyxoviridae* and order of *Mononegavirales*. The genomes of avian paramyxoviruses encode six proteins from 5′ to 3′: nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin-neuraminidase (HN) and RNA-dependent RNA polymerase (L). Two additional proteins, V and W, are expressed by RNA-editing of the P gene with insertion of +1 and +2 nucleotides, respectively (Steward *et al.*, 1993). The 3′ and 5′ ends of the each gene contain short extragenic sequences known as the 'leader' and 'trailer' regions, respectively. The viral RNA polymerase begins transcription at the 3′ end and proceeds downstream in a sequential manner generating individual mRNAs by a start–stop mechanism guided by gene-start (GS) and gene-end (GE) signals that flank each gene. Non-coding intergenic sequences (NCS) are present between gene boundaries and are not copied into the mRNAs. During RNA replication, the GS and GE signals are ignored and a complementary copy of the genome (the anti-genome) is synthesized, which serves as the template for synthesis of the progeny genome (Lamb *et al.*, 2005).

The APMV-1's virion is composed of RNA binding nucleocapsid cores that bind to the P and L proteins shortly after synthesis to form the ribonucleoprotein (RNP) complex. This complex becomes the template for transcription by L protein. The L protein binds the genomic RNA at the 3′ end of the RNP complex and transcribes the genes using a start–stop mechanism. The genome of APMV-1 is always 15,186, 15,192 or 15,198 nucleotides (nt) in length and follows the so-called 'rule of six', which is essential for viral replication (Kolakofsky *et al.*, 2005).

The virion is enveloped and includes the non-glycosylated M protein and two types of glycosylated envelope proteins, which both have key roles in the pathogenesis of APMV infection: an attachment protein HN, which is capable of both haemagglutination and neuraminidase activities, and F protein (Lamb and Kolakofsky, 2001). The interaction of the HN and F proteins promotes the fusion of the viral and host cell membranes (Huang *et al.*, 2004). HN is responsible for the virus binding to sialic acid (SA) receptors at the cell surface of the host cells bringing the F protein closer to the host cell, whereas the F protein mediates the fusion of the viral envelope with the plasma membrane of the host cell. Fusion of the membranes allows the viral genome to enter the host cell, where initiation of viral replication occurs (Lamb *et al.*, 2006). The exact function of the neuraminidase activity of the HN protein during virus replication is not completely understood. The neuraminidase activity of HN protein is likely to assist in virus release from infected cells by removing the cell receptors from the host cells, which prevents reattachment of the released virus particles and virus aggregation.

F protein is synthesized as a biologically inactive precursor (F0). Proteolytic cleavage of the F protein into two functional F1 and F2 polypeptides by cellular protease is essential for infectivity of progeny virions and is a key determinant of pathogenicity among paramyxoviruses. The specific nature of the cleavage process and the characteristics of the F0 protein differ among different avian paramyxoviruses. However, the APMV-1 can be crudely divided into two groups: those with a single basic amino acid at the cleavage site and those with multiple basic amino acids at the cleavage site. The F0 protein cleavage site of the virulent form of APMV-1 contains a furin recognition site with multiple basic amino acids (arginine or lysine) surrounding the glutamine at position 114 (C-terminus of the F2 subunit) and a phenylalanine at position117 (N-terminus of the F1 subunit). Host ubiquitous intracellular proteases are able to cleave the F0 protein in the trans-Golgi network because of the presence of the polybasic amino acids; on arrival at the plasma membrane these F proteins are already in the active state.

Low virulence forms of the virus have a single basic residue at the cleavage site

and a leucine at position 117, and the F0 proteins remain in the inactive state when they reach the plasma membrane. These viruses are only activated by extracellular proteases in epithelial cells of, principally, the respiratory and gastrointestinal tracts. This limited 'cleavability' restricts infectivity of the virus to fewer species of birds and significantly reduces the pathogenic potential of these viruses (Leeuw *et al.*, 2005; Lamb *et al.*, 2006).

The genetic diversity of APMVs is still largely unexplored, as hundreds of avian species have never been surveyed for the presence of viruses that do not cause significant signs of disease or are not economically important. However, improved molecular techniques for rapid nucleotide sequencing have resulted in the availability of sequence data for increasing numbers of APMV-1 viruses in the GenBank database. Due to use of relatively short sequence length of the F gene to predict virulence and to assess the local spread and global epidemiology of APMV-1 viruses, molecular approaches have been of enormous importance in recent years. Although considerable genetic diversity has been noticed between different strains of APMV-1, viruses sharing temporal, geographical or antigenic characteristics tend to fall into the same specific clades (Miller *et al.*, 2009).

Two different classification models for APMV-1 are used to group isolates based on genetic characteristics of the genes, particularly the F gene (Lomniczi *et al.*, 1998; Czegledi *et al.*, 2002; Aldous *et al.*, 2003). Differences in groupings arise between the two classification methods and either can be used based on preference. One classification proposed by Aldous *et al.* (2003) was based on genotypes or genetic lineages grouped under serotype 1 (APMV-1). This grouping model divides APMV-1 into six lineages (lineages 1 to 6). Sub-lineages (a–d) were created in lineages 3 and 4, while sub-lineages (a–e) were formed in lineage 5. These genetic groupings are indicated by lineage and sub-lineage such as 3a and 3b. A second classification method was based on the genomic characterization and sequence analysis of the F and later L genes group isolates into either Class I or Class II as opposed to lineages (Lomniczi *et al.*, 1998; Kim *et al.*, 2008). Isolates from Class I are present in the live bird markets in Hong Kong and USA, domestic poultry and wild waterfowl. Class I is composed of primarily low virulent isolates, but one virulent isolate has been included in that classification. Class I viruses have a worldwide distribution and are further divided into nine genotypes. Isolates grouped in Class I have the longest APMV-1 genome at 15,198 nucleotides. Class I isolates are not usually reported to the OIE because of their low virulence designation. Isolates causing all four panzootics from 1920 to the present are classified as Class II. Class II viruses are usually recovered from poultry, pet birds and wild waterfowl. Class II viruses are further divided into genotypes I through IX. Genotypes I through IV and IX have slightly shorter genome lengths at 15,186 nucleotides. These genotypes are considered 'early' because of their identification between 1930 and 1960. Genotypes V through VIII and X have a medium length genome of 15,192 nt and are considered 'late' because of their identification after 1960. All NDVs are classified as Class II, except for one isolate, which caused the Australian outbreak from 1998 to 2000 (Miller *et al.*, 2010b). Besides these, there are increasing reports of the emergence of novel genotypes/subgenotypes in many regions of Africa and Asia, including our recent report from Pakistan (Munir *et al.*, 2012a, 2012b). However, the classification of APMV-1 and the so-called new genotype are still under debate (Miller *et al.*, 2010b).

2.4 Viral Replication

Avian paramyxoviruses replicate in the cytoplasm of the infected cells. The attachment of the virus to the cell receptors, mediated by the HN protein, initiates the first step of viral replication. Following attachment, the function of the F protein mediates fusion of the viral and cell membranes, allowing the viral nucleocapsid complex to enter the host cell.

The transcription of the viral negative-sense RNA is initiated at a single promoter site within the 3′ leader region of the viral RNA by a sequential interrupted start–stop mechanism to yield a series of 6–8 subgenomic mRNAs by transcription. The termination–re-initiation process controls the synthesis of mRNA; this process is controlled by the GS signal that signals transcription initiation and encodes the 5′ end of each mRNA, and the GE signal that directs polyadenylation and termination of the mRNA at the 3′ end. The virus uses the host-cell translation machinery to initiate viral protein synthesis. The viral proteins synthesized in an infected cell are then transported to the cell membrane. The host-cell membrane becomes modified by the incorporation of the viral protein to form the new viral envelope. Following the alignment of the RNP complex in close proximity to modified regions of the cell membrane, the new virus particles are released by budding from the cell surface.

2.5 Pathogenesis

Multiple factors including host species, age, immune status, secondary infections, stress, environmental conditions, the amount of virus transmitted and the route of transmission affect the pathogenicity of the virus, but the most important factor is the strain of the infecting virus. Viruses infect the epithelial cells of the respiratory and the gastrointestinal tract (Cheville *et al.*, 1972). In these organs, the virus is spread by ciliary action and cell-to-cell infection. The subsequent spread of virus in infected hosts largely depends on strain virulence. Less virulent strains are present at low titres in the respiratory and intestinal tracts, while more virulent strains spread rapidly to several other organs like kidneys, lungs, bursa and the spleen; very virulent virus can be found in all tissues.

Viral entry proteins are considered to be major virulence determinants, most importantly the amino acid motif at positions 112–117 of the cleavage site of F0 protein, which determines whether the virus will be restricted to respiratory and gastrointestinal tissues or able to cause systemic infection (Alexander, 2003; Leeuw *et al.*, 2005; Lamb *et al.*, 2006). Besides the characteristics of the F protein, the length of the HN protein has also been associated with the pathogenicity of avian paramyxoviruses (Lamb *et al.*, 2006). The HN_0 precursor protein is composed of 616 amino acid residues in avirulent strains of APMV-1. This inactive HN_0 is converted to an active protein by proteolytic cleavage of a small glycosylated fragment at the 3′ end of the protein (Gorman *et al.*, 1988). The open reading frame of other APMV-1 strains, including lentogenic, mesogenic and velogenic strains includes stop codons located before the HN_0 stop codon, resulting in active proteins of 571 and 577 amino acids in length. It is of interest to note that, to date, only the shortest HN protein (571 aa) has been found in velogenic strains (Alexander, 2003). Thus, shortening of the HN active protein plays some role in virulence but is not completely understood.

2.6 Immune Evasion

Many viruses have evolved mechanisms to evade or antagonize the innate immune response of its host (Samuel, 2001). This phenomenon has also been well documented for paramyxoviruses, which have evolved mechanisms to escape or prevent both interferon (IFN) production and IFN responsive signal transduction. In many cases, the IFN evasion activities of paramyxovirus are mediated by the virus-encoded V protein. The paramyxovirus V protein is derived from the polycistronic P gene (Steward *et al.*, 1993) and is characterized by a cysteine-rich C-terminal domain that is highly conserved among several paramyxovirus species. *In vitro* studies of an NDV mutant that completely or partially lacks the V protein or contains a mutated V protein show severe growth impairment (Mebatsion *et al.*, 2001). It has been shown that viruses with mutated V proteins, in contrast to parental viruses, are unable to degrade the STAT1 protein (Huang *et al.*, 2003), which is an important element of

the IFN signaling pathway (Haller *et al.*, 2006). These results show that the V protein plays an important role in NDV pathogenicity (Park *et al.*, 2003).

2.7 Clinical Signs

Clinical symptoms generally depend on the age and immune status of the host, the route of infection and the virulence of the strain. In addition, the clinical signs produced by specific viruses may differ dramatically between different species of birds. In general, chickens and turkeys are most susceptible to infection with APMV-1, but the clinical signs could be less severe in turkeys and the disease signs are mainly respiratory-based. In waterfowl, such as ducks and geese, the APMV-1 infections are predominantly symptomless. However, several outbreaks of severe disease in ducks and geese have been reported from South East Asia (Liu *et al.*, 2003; Wan *et al.*, 2004; Jinding *et al.*, 2005). Mallards experimentally infected with a velogenic strain of APMV-1 showed severe clinical symptoms like leg paralysis, torticollis and wing droop (Friend and Trainer, 1972). Reports on clinical manifestations following natural infection of wild birds are few; although many species of birds are susceptible to infection with APMV-1, wild birds are generally considered to be resistant to clinical signs. However, large-scale illness and death from APMV-1 infection in wild birds has been reported in double-crested cormorants (*Phalacrocorax auritus*) in Canada and the USA. In field conditions, the clinical signs were observed only in sick juvenile double-crested cormorants, and included torticollis or twisting of the head and neck, ataxia or a lack of muscular coordination, tremors, paresis or incomplete paralysis, including unilateral or bilateral weakness of the legs and wings, and clenched toes (Wobeser *et al.*, 1993; Meteyer *et al.*, 1997).

The general symptoms include: loss of appetite; respiratory distress (gasping); cyanosis of the comb; oedema of the head, face and wattles; a sudden decrease in egg production sometimes in combination with depigmentation and loss of egg shell and egg quality in layer hens; poor body condition; reduced feed intake and ruffled feathers. Green diarrhoea is frequently seen in birds that do not die early in infection.

Strains of APMV-1 can be differentiated into pathotypes on the basis of clinical signs in chickens. The most virulent form (velogenic [MDT (mean death time) <60 h]) is characterized as acute disease that can be present in birds of all ages and case fatality can reach 100%. The disease appears suddenly, resulting in high mortality in the absence of other clinical symptoms. In contrast, clinical manifestations of strains with modest virulence (mesogenic, MDT 60–90 h) are strongly dependent on the age of animals. In chicks, morbidity within a flock is as high as 100%, but in adult healthy hens this rarely reaches 50%. Hence, case fatality varies between 5% and 50%. Mesogenic strains of APMV-1 usually cause respiratory disease in infected birds. Even more pronounced is the age-dependent manifestation of NDV infection with lentogenic pathotypes. In fully susceptible young birds, serious respiratory symptoms can be seen that can result in mortality in combination with infection by other microorganisms like *Mycoplasma gallisepticum* (Alexander, 2003).

2.8 Gross Lesions

As for the symptoms (the pathological findings), the gross lesions and organs involved vary depending on the characteristics of the infecting virus, in addition to the host and environmental factors that may affect the severity of the disease. A virulent virus causes predominantly haemorrhagic lesions in various parts of the intestinal tract, particularly in the mucosa of the proventriculus, caeca and small intestine of the infected birds. In very severe cases, haemorrhages are also found in sub-cutis, muscles, larynx, trachea, lungs, airsacs, pericardium and myocardium. In adult hens, the ovarian follicles are often flaccid and degenerative. Necrotic foci are also observed in the pancreas. In contrast, gross pathogenic changes

are not found in the central nervous system of birds infected with NDV, regardless of the pathotypes of the strain and the species involved. Mucosal haemorrhage and marked congestion of the trachea and lung may be observed in the respiratory tract. Airsacculitis of varying severity, often in association with secondary infections, occurs even after infection with relatively low virulence strains.

2.9 Transmission

The information available on the transmission of avian paramyxoviruses is limited to the transmission of APMV-1 between domestic birds. The replication of the virus in either the respiratory or intestinal tract of newly infected birds allows for spreading of the virus through nasal discharge or in the faeces. The primary route of transmission is either by inhalation of large or small droplets liberated from infected birds or by the ingestion of faecal-contaminated material. The incubation period is 7–14 days, with the virus being shed in the faeces as early as 2 days post-infection (Alexander, 2003). Infection may spread directly during the incubation period or indirectly through material contaminated by excretions or secretions from infected birds. The ability of such virus transmission, however, depends on environmental factors such as temperature and humidity, as well as the viral load contained in the contaminated material.

True vertical transmission (i.e. passing of the virus from parents to progeny via the embryo) is not likely to occur as a result of infections with virulent strains, but its significance in epidemics of low-virulence APMVs remains unclear. Capua *et al.* (1993) reported the findings of commercial embryonated hens' eggs contaminated with virulent APMV virus and reported no problems of embryo mortality or reduced hatchability. The virus could also be isolated from young chickens hatched from these contaminated eggs without showing any typical clinical signs of NDV. The eggs originated from a laying flock with no clinical signs of NDV, but despite their high levels of antibody, virulent APMV virus was isolated from cloacal swabs collected from the laying flock (Capua *et al.*, 1993).

The airborne spread of virus by wind in droplets, dust or dried faeces has been considered to be important in the case of APMV-1, but its significance varies considerably, and is influenced by air temperature and humidity (Alexander, 2003). Years of experience of outbreak management strongly indicate that the greatest potential for the spread of disease is by the actions of humans, including the mechanical spread of infective material preliminarily by movements of people and equipment or by the movement of infected birds between farms.

Pigeons can be infected by classical NDV (cNDV) strains and by so-called 'pigeon' APMV-1 (pPMV-1) strains. The pPMV-1 strains most probably appeared originally in the Middle East or North-East Africa in the 1970s (Kaleta *et al.*, 1985) from where they spread by racing pigeons to Europe in the early 1980s and were first diagnosed in Italian racing pigeons in 1981 (Vindevogel *et al.*, 1982). The pPMV-1 strains spread throughout Europe in 1983 (Alexander *et al.*, 1984) and gave rise to an on-going panzootic outbreak (Aldous *et al.*, 2004). Between 2000 and 2009, a staggering 1364 pPMV-1 cases, in mostly domestic pigeons, were reported from EU countries (Alexander *et al.*, 2011). In pigeons, pPMV-1 infection is associated with severe neurological involvement. The incubation period lasts from 4–6 days to 3–4 weeks. Morbidity ranges from 30% to 70%, but mortality may reach as high as 40%. The pigeon variant of NDV normally does not produce respiratory signs in infected birds and necropsy findings are non-specific. The virus is spread in the faeces only during the acute phase of the disease, but may persist in the lungs and brain for 4 and 5 weeks, respectively, although recovered birds do not become carriers. The virus typically gains virulence from serial passage in susceptible gallinaceous hosts (Dortmans *et al.*, 2011). The prevailing view is that, despite vaccination, domestic pigeons constitute the major reservoir, from which occasional spread occurs

to feral pigeons and poultry. Feral pigeons are descendants of domesticated rock pigeons and are globally abundant in cities and agricultural areas, often achieving a pet's status. There is a remarkable lack of information on pPMV-1 occurrence in feral pigeons, which is further aggravated by the fact that these birds are rarely the subject of population ecology studies and wildlife disease surveillance. Hence, information on local bird movements, population sizes, preferred habitats and population-limiting factors, e.g. diseases, are lacking.

The belief that pPMV-1 strains do not infect poultry readily has been challenged recently. Between 2000 and 2007, 12 pPMV-1 outbreaks in poultry were reported in EU countries. Outbreaks in poultry typically occur in naïve flocks and many cases may go unnoticed (Alexander *et al.*, 2011).

2.10 Avian Paramyxoviruses Other Than APMV-1 (NDV)

Very little is known about the molecular and biological characteristics, and pathogenicity, of APMV serotypes 2–10. Only APMV types 2, 3, 6 and 7 have been associated with disease in domestic poultry (Tumova *et al.*, 1979a; Bankowski *et al.*, 1981; Redmann *et al.*, 1991; Zhang *et al.*, 2006). These viruses cause respiratory disease and egg production losses, which may be severe when exacerbated by other infections or environmental stresses. Because of the absence of distinct clinical signs and symptoms in domestic poultry, it is difficult to detect a representative strain of APMV-2–10 infections in field conditions. The lack of proper diagnostic tests against these serotypes hinders the investigation of their epidemiology and economic impact on the poultry industry. The branching pattern for all the APMVs is shown in Fig. 2.1.

2.10.1 APMV-2

The first strain of APMV-2 was isolated in 1956 in Yucaipa, California from a diseased chicken suffering from infectious laryngotracheitis virus (Bankowski and Corstvetre, 1960). The virus was shown to be serologically

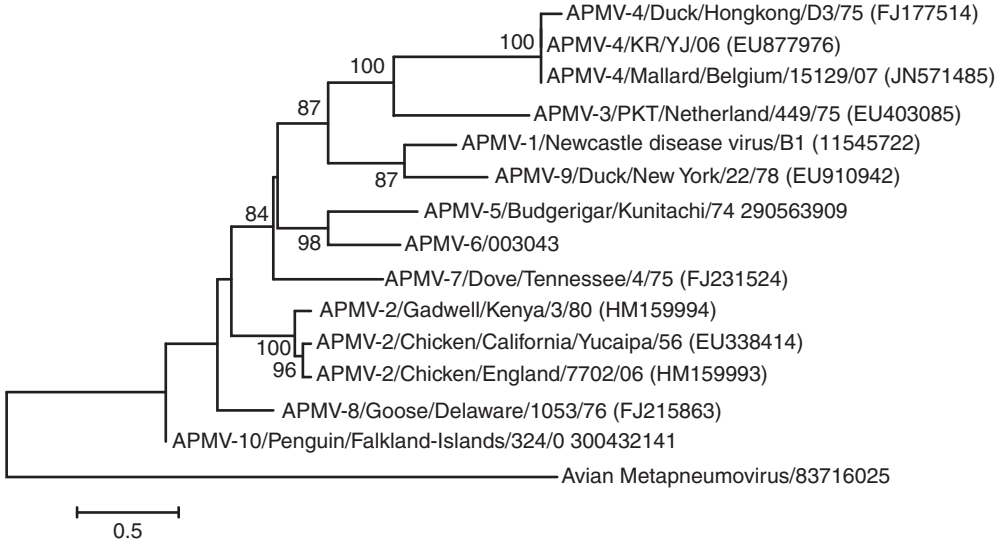

Fig. 2.1. Phylogenetic analysis of AMPV 1–10 based on complete genome sequences.

distinct from APMV-1 and caused only mild respiratory disease in chickens (Alexander, 1980). Bankowski *et al.* (1981) reported that natural, as well as artificial, exposure of laying turkeys to APMV-2 caused a pronounced decline in hatchability and poultry yield. Many APMV-2 strains have since been isolated from chickens, turkeys and feral birds across the globe (Fleury and Alexander, 1979; Goodman and Hanson 1988; Zhang *et al.*, 2006). APMV-2 infections have been reported in chickens in the USA, Canada, Russia, Japan, Israel, India, Saudi Arabia and Costa Rica, and in 2006 in Scotland, and in turkeys in the USA, Canada, Israel, France and Italy (Wood *et al.*, 2008). High prevalence of APMV-2 antibodies has been reported in breeder, layer and broiler chickens in several countries, including the USA (Bankowski *et al.*, 1968), Spain (Maldonado *et al.*, 1995) and China (Zhang *et al.*, 2007). However, the infection appeared to be more prevalent in turkeys than in chickens (Bankowski *et al.*, 1968). APMV-2 viruses have been frequently isolated from passerine and psittacine birds (Senne *et al.*, 1983). The Bangor strain of APMV-2 was isolated from a finch during a routine quarantine evaluation, and the biological and serological characterization suggested that the Bangor strain might represent a subgroup within serotype 2 (McFerran *et al.*, 1973). Despite the global distribution and prevalence of APMV-2 in different bird species, limited information is available regarding the serological and genetic relationships among these strains. Recently, based on genetic and antigenic characteristics of APMV-2 isolates, two distinct subgroups of APMV-2 (Bangor and California/Yucaipa) were proposed (Subbiah *et al.*, 2010). These two subgroups differ substantially in their nucleotide and amino acid sequences but show only discrete antigenic differences. The genome lengths of APMV-2 strains Bangor, England and Kenya isolated from a finch, a chicken and a gadwell duck, respectively, are 15,024, 14,904 and 14,916 nt, respectively, compared with 14,904 nt for the prototype strain of APMV-2s: Yucaipa (Subbiah *et al.*, 2008). Interestingly, despite these variations, each genome consists of six non-overlapping genes in the order of 3′-N-P/V/W-M-F-HN-L-5′, with a 55-nt leader at the 3′ end. The length of the trailer at the 5′ end of the Bangor strain is 173 nt, compared with 154 nt for the strains England, Kenya and Yucaipa. Furthermore, Bangor has a single basic aa residue (^{101}TLPSAR↓F^{108}) at the fusion protein cleavage site compared with the dibasic aa (^{93}DKPASR↓F^{100}) found in the other three strains (Subbiah *et al.*, 2010).

2.10.2 APMV-3

APMV-3 was first isolated from a turkey in Ontario, Canada in 1967, and in Wisconsin, USA in 1968. Since then, many APMV-3 strains have been isolated from turkeys in different parts of the world (Tumova *et al.*, 1979b, Macpherson *et al.*, 1983). Recently, APMV-3 was isolated from ostrich, indicating a wide host range for the virus (Kaleta *et al.*, 2010). The APMV-3 strain parakeet/Netherlands/449/75, isolated from parakeets in The Netherlands, is the prototype for the entire serotype (Alexander and Chettle, 1978). APMV-3 was also frequently isolated from captive cage birds such as *Psittaciformes* and only rarely from *Passeriformes* (Alexander, 1980). APMV-3 has been associated with encephalitis and high mortality in caged birds, and with respiratory disease including coughing, nasal discharge and swelling of the infra-orbital sinus in turkeys (Tumova *et al.*, 1979a). The virus causes acute pancreatitis and central nervous system symptoms in *Psittaciformes* and *Passeriformes* (Beck *et al.*, 2003). APMV-3 also infects chickens at an early age, with evidence of stunted growth that may be more marked in broiler chicken breeds (Alexander and Collins, 1982).

The exact economic impact of APMV-3 infection in the poultry industry is not known. This is partly because the pathogenicity of APMV-3 in poultry species is not well studied. Serological studies indicate the existence of at least two subgroups among APMV-3 isolates. Isolates from psittacines have been shown to be more

closely related to each other than to American and European turkey isolates (Alexander *et al.*, 1984).

Although there is a high degree of amino acid sequence variation between APMV-3 and APMV-1, by the HI test there is cross-reaction between APMV-3 and APMV-1 serum samples, which often leads to misdiagnosis of APMV-3 as APMV-1. Early studies by Alexander *et al.* (1979a) showed that chickens infected with APMV-3 viruses were protected against challenge with virulent APMV-1.

The genome of the prototype strain of APMV-3 was found to be the longest among the APMVs and has the longest trailer region in the family *Paramyxoviridae*. The genome is 16,182–16,272 nt in length, consisting of six non-overlapping genes in the order of 3′-N-P/V/W-M-F-HN-L-5′, with a 55-nt leader at its 3′ end and a 681-nt trailer at its 5′ end, along with intergenic regions of 31–63 nt. The cleavage site of F protein, ARPRGR↓L, does not conform to the preferred cleavage site of the ubiquitous cellular protease furin (Kumar *et al.*, 2008, 2010).

2.10.3 APMV-4

Initial strains of APMV-4 were isolated from hunter-killed wild mallard, gadwall and wood ducks on the Mississippi Flyway in the USA (Webster *et al.*, 1976) and from chickens, ducks and geese in Hong Kong during poultry influenza surveillance programmes (Shortridge and Alexander, 1978). Apart from an isolate from captive ringed teals suffering from haemorrhagic enteritis (Gough and Alexander, 1984), all other viruses of the APMV-4 subtype have been isolated from wild waterfowl showing no clinical signs of disease and are found to be widely distributed among waterfowl throughout the world (Yamane *et al.*, 1982; Tumova *et al.*, 1989; Stanislawek *et al.*, 2002; Jeon *et al.*, 2008; Rosseel *et al.*, 2011). Infections of poultry with this subtype have generally been limited to ducks and geese, with the exception of occasional isolation of APMV-4 viruses from chickens in Hong Kong that probably represent limited spread from wild waterfowl (Shortridge and Alexander, 1978; Alexander *et al.*, 1979b).

The whole genome sequences of three representative strains of APMV-4 have been reported (Jeon *et al.*, 2008; Nayak *et al.*, 2008; Rosseel *et al.*, 2011). The genome of APMV-4 is 15,054 nt in length and the maximum genetic distance within the APMV-4 serotype ranges from 2 to 8% nucleotide sequence distance, confirming earlier findings by Alexander *et al.* (1979b), who found that there is no major antigenic variation between representative isolates of APMV-4 from different geographical locations. The genome contains six non-overlapping genes in the order 3′-N-P/V-M-F-HN-L-5′. The intergenic sequences vary in length from 9 to 42 nt. The genome contains a 55-nt leader region at the 3′ end and 17-nt at the 5′ trailer region, which is the shortest in the *Paramyxoviridae* family.

Analysis of mRNAs transcribed from the P gene showed that 35% of the transcripts were edited by the insertion of one non-templated G residue at an editing site leading to the production of V mRNAs. No mRNA was detected that contained an insertion of two non-templated G residues, indicating that the W mRNAs are inefficiently produced in APMV-4 infected cells. A monobasic fusion protein cleavage site (DIPQR↓F) was observed in APMV-4 viruses; however, exogenous proteases are not required for the growth of APMV-4 in cell culture, indicating that the cleavage does not depend on a furin site (Nayak *et al.*, 2008).

Experimental infection of chickens with APMV-4 and APMV-6 showed mild interstitial pneumonia, catarrhal tracheitis and BALT or GALT hyperplasia, suggestive of viral disease (Warke *et al.*, 2008). Kim *et al.* (2012) studied the growth properties of prototype strains of APMV serotypes 2–9 in cell culture and in chickens and ducks, and revealed limited replication of APMV-4 (APMV-4/duck/Hong Kong/D3/75) in chickens both *in vitro* and *in vivo*, suggesting that APMV-4 has a strong host range restriction in chickens.

2.10.4 APMV-5

APMV-5 was first isolated from an epizootic outbreak involving budgerigars (*Melopsittacus undulatus*) in Kunitachi, Tokyo, Japan in 1974 (Nerome *et al.*, 1978). This virus was serologically and antigenically distinct from the other previously known APMV serotypes and serves as the prototype virus for this serotype. APVM-5 causes a disease in budgerigars that is characterized by depression, dyspnoea, diarrhoea, torticollis and acute fatal enteritis in immature budgerigars, leading to very high mortality. A second outbreak occurred among budgerigars in Brisbane in Queensland, Australia. Experimental infection of APMV-5 isolated from this outbreak caused no signs of disease in young and adult chickens and pigeons, but caused acute fatal enteritis among immature budgerigars (Mustaffa-Babjee *et al.*, 1974). The third known outbreak of APMV-5 disease occurred in budgerigars in the UK in 1993 (Gough *et al.*, 1993). The clinical signs consisted mainly of vomiting and diarrhoea followed by death. Of all of the APMVs examined to date, only APMV-1 and APMV-5 have been associated with 100% mortality (Nerome *et al.*, 1978). APMV-5 appears to differ from all the other APMV serotypes in two major attributes: (1) the inability to grow in the allantoic cavity of embryonated chicken eggs and (2) the failure to cause haemagglutination with chicken RBC (Nerome *et al.*, 1978). However, the virus grew in the amniotic cavity of embryonated chicken eggs and in different established cell lines like Vero and chicken embryo fibroblast cell lines (Nayak *et al.*, 2011; Kim *et al.*, 2012).

The genome is 17,262-nt long, which is the longest among members of the genus *Avulavirus*, and encodes six genes in the order of 3′-N-P/V/W-M-F-HN-L-5′ with inter-genic regions of 4–57 nt. The genome contains a 55-nt leader sequence at the 3′ end and a 552-nt trailer sequence at the 5′ end. The P gene contains a conserved RNA editing site and is predicted to encode the P, V and W proteins. The cleavage site of the F protein of APMV-5 (GKRKKR↓F) is the only example from APMV-2–9 to have a furin motif and conforms to the cleavage site motif of the ubiquitous cellular protease furin. Consistent with this, exogenous protease was not required for virus replication *in vitro*. However, the intra-cerebral pathogenicity index of APMV-5 strain Kunitachi in 1-day-old chicks was found to be zero, indicating that the virus is avirulent for chickens despite the presence of a polybasic F cleavage site (Samuel *et al.*, 2010).

There are no reports of isolation of APMV-5 from poultry. Experimental infection of 2-week-old chickens by the oculonasal route with prototype strain of APMV-5 resulted only in low antibody response in just half of the inoculated animals. No antigen was detected in any tissues harvested from chickens infected with APMV-5 and no clinical signs were observed in any chickens (Nayak *et al.*, 2011). Thus, although APMV-5 bears a furin cleavage site and can cause 100% mortality in budgerigars (Nerome *et al.*, 1978), it was completely avirulent and replicated inefficiently in chickens. This is suggestive of a host range difference that was not greatly ameliorated by the presence of a multi-basic cleavage site.

2.10.5 APMV-6

APMV-6 strains have been isolated from a wide range of avian species from different parts of the world (Shortridge *et al.*, 1980; Marius-Jestin *et al.*, 1987; Chang *et al.*, 2001; Stanislawek *et al.*, 2002). The first strain of APMV-6 was isolated from a domestic duck in Hong Kong in 1977 (APMV-6/duck/Hong Kong/18/199/77), and was reported to be non-pathogenic in experimentally infected chickens (Shortridge *et al.*, 1980). A single report of an APMV-6 infection leading to mild respiratory disease and egg production problems has been reported in turkeys in Canada (Alexander, 2003). Little is known about the antigenic and genetic variation among APMV-6 viruses. Initial studies on several isolates of APMV-6 from various geographical locations showed no significant serological variations among these isolates (Shortridge

et al., 1980; Lipkind and Shihmanter, 1986). However, recent genetic studies revealed the existence of at least two classes within the APMV-6 subtype. The APMV-6 class I isolates representing distant geographical locations differed by less than 8% from each other, but differed by about 30% from the single class II isolate IT4524-2 (Chang *et al.*, 2001; Xiao *et al.*, 2010; Rasseel *et al.*, 2011). Only six complete nucleotide sequences of APMV-6 serotypes have been so far reported to GenBank. The 16,236-nt genome encodes the NP, P/V/W, M, F, SH (small hydrophobic protein), HN and L proteins from 3′ to 5′. Interestingly, APMV-6 contains a gene that encodes the SH protein, which is absent in other APMVs, but the biological function of the APMV-6 SH protein in not known (Chang *et al.*, 2001). However, the SH proteins of the rubulaviruses, simian virus type 5 and mumps virus appear to play essential roles in blocking the TNF-alpha-mediated apoptosis pathway (Wilson *et al.*, 2006). Although the F protein of APMV-6 strains has a monobasic cleavage site (PEPR↓L), replication of APMV-6 in cell culture does not require the addition of exogenous trypsin (Kim *et al.*, 2012).

2.10.6 APMV-7

APMV-7 was first isolated from a hunter-killed dove (*Columba* species) in 1975 in Tennessee, USA (Alexander *et al.*, 1981). A few years later, the same serotype of APMV was isolated from pigeons and doves in the UK and Japan. APMV-7 was isolated from the pooled intestinal contents of two 5-month-old sick ostriches (*Struthio camelus*) showing anorexia and depression in 1996 in California (Woolcock *et al.*, 1996), and was also associated with a natural outbreak of respiratory disease in commercial turkey breeder flocks in Ohio in 1997 (Saif *et al.*, 1997). APMV-7 infection in turkeys caused respiratory disease, mild multifocal nodular lymphocytic airsacculitis and decreased egg production (Saif *et al.*, 1997). Serological comparisons of the prototype strain of APMV-7 (dove/Tennessee/4/75) together with several other isolates representing the same serotypes have shown considerable antigenic variation, placing the studied viruses in three groups (Alexander *et al.*, 1991).

Recently, Xiao *et al.* (2009) determined the complete genome sequence of prototype strain dove/Tennessee/4/75. The genome size of APMV-7 appeared to be 15,480-nt long and contains six non-overlapping genes in the order of 3′-N-P/V/W-M-F-HN-L-5′. The 3′-leader and 5′-trailer sequences of the genome are 55- and 127-nt long, respectively. The putative APMV-7 F protein cleavage site (PSSR↓F) contains a single basic residue (R) and phenylalanine (F) residue at the N-terminus of F1 subunit, and also grew in cell culture without supplementation of exogenous protease. Nevertheless, the virus grew in only a few established cell lines, indicating a restricted host range (Xiao *et al.*, 2009).

2.10.7 APMV-8

APMV-8 strain goose/Delaware/1053/76 was first isolated in 1976 from a feral Canada goose (*Branta canadensis*) in the Atlantic Flyway in the USA (Cloud and Rosenberger, 1980). APMV-8 strain pintail/Wakuya/20/78 was isolated from a feral pintail duck (*Anas acuta*) in Wakuya, Japan in 1978 (Yamane *et al.*, 1982). No major antigenic variations have been shown between viruses isolated in the USA and Japan and there are no reports of the isolation of APMV-8 from poultry. Complete genome sequence analysis of the strains goose/Delaware/1053/76 (prototype strain) and pintail/Wakuya/20/78 were presented by Paldurai *et al.* (2009). The genome of each strain is 15,342-nt long and consists of six genes in the order of 3′-N-P/V/W-M-F-HN-L-5′. The genes are flanked on either side by conserved transcription start and stop signals, and have inter-genic regions ranging from 1 to 30 nt. The genome contains a 55-nt leader region at the 3′ end and a 171-nt trailer region at the 5′ end. Both strains grew in embryonated chicken eggs and in primary chicken embryo kidney cells, and 293T cells. Both strains contained

only a single basic residue at the cleavage activation site of the F protein and their efficiency of replication *in vitro* depended on, and was augmented by, the presence of exogenous protease in most cell lines (Paldurai *et al.*, 2009).

2.10.8 APMV-9

APMV-9 strains have been isolated from ducks in several countries (Sandhu and Hinshaw, 1981; Capua *et al.*, 2004). The APMV-9 strain PMV-9/domestic duck/New York/22/78 was initially isolated from a domestic duck during a routine surveillance programme in New York (Sandhu and Hinshaw, 1981). The genome of strain PMV-9/domestic duck/New York/22/78 consists of 15,438 nt and encodes six non-overlapping genes in the order of 3′-N-P/V/W-M-F-HN-L-5′ with inter-genic regions of 0–30 nt. The genome length contains a 55-nt leader sequence at the 3′ end and a 47-nt trailer sequence at the 5′ end. The virus required exogenous protease for *in vitro* replication and grew only in a few established cell lines, indicating a restricted host range (Samuel *et al.*, 2009).

2.10.9 APMV-10

The biological, serological and genomic characterization of a paramyxovirus recently isolated from Rockhopper penguins (*Eudyptes chrysocome*) (Miller *et al.*, 2010a) and Magellanic penguins (*Spheniscus magellanicus*) (Fornells *et al.*, 2012) suggested that this virus represented a new avian paramyxovirus serotype, APMV10. Sequence data produced using random priming methods revealed a genomic structure typical of APMV. The nucleotide lengths of the complete genome of the prototype strain of APMV-10 (APMV10/penguin/Falkland Islands/324/2007) were 15,226 nt. This is longer than those of APMV-1 (15,186 nt) (Krishnamurthy and Samal, 1998), APMV-2 (14,904 nt) (Subbiah *et al.*, 2008) and APMV-4 (15,054 nt) (Nayak *et al.*, 2008; Jeon *et al.*, 2008), but is shorter than those of APMV-3 (16,272 nt) (Kumar *et al.*, 2008), APMV-5 (17,262 nt) (Samuel *et al.*, 2010), APMV-6 (16,236 nt) (Chang *et al.*, 2001), APMV-7 (15,480 nt) (Xiao *et al.*, 2009), APMV-8 (15,342 nt) (Paldurai *et al.*, 2009) and APMV-9 (15,438 nt) (Samuel *et al.*, 2009). Miller *et al.* (2010a) demonstrated the inability of APMV-10 to grow in Vero and DF1 cells without the addition of trypsin. Phylogenetic evaluation of the APMV-10 viruses in relation to other APMVs revealed that the sequences of APMV-10 were most closely related to APMV-2 and APMV-8 (Miller *et al.*, 2010a; Fornells *et al.*, 2012).

Recently, Kim *et al.* (2012) evaluated the *in vitro* (growth kinetics and cytopathic effect in chicken fibroblast cells) and *in vivo* replication and tropisms for prototype strains of APMV serotypes 2–9 by infecting prototype strains of each serotype in two different ages of chickens (1-day-old and 2-week-old chickens) and 3-week-old ducks. The study indicates that APMV serotypes 2–9 were avirulent in both chickens and ducks as well as in standard international assays like the MDT assay in chicken eggs and the intra-cerebral pathogenicity index (ICPI) test in 1-day-old SPF chickens. Each of the APMV serotypes replicated to low-to-moderate titres in the trachea of chickens, with APMV-3 having the highest titres. Among APMV types 2–9, only APMV-3 replicated systemically and was neuroinvasive and neurotropic, although it was not neurovirulent. In another study, Nayak *et al.* (2012) evaluated the effect of prior infection of chickens with APMV-2–9 on the replication and pathogenicity of virulent NDV challenge. By using convalescent antiserum on each serotype of APMV they could demonstrate that most of the serotypes 2–9 have little or no serologic cross-reactivity with PMV-1. There was significant cross-reactivity with APMV-1 only in the case of APMV-3, and only by HI and neutralizing assays. Somewhat unexpectedly, the extent of sequence relatedness between NDV and a different APMV serotype did not predict the level of cross-protection. For example, NDV is most closely

related to APMV-9 by genome nucleotide sequence (60%) as well as the amino acid sequences of the protective and neutralization antigens F (54.4%) and HN (62%), but APMV-9 provided only a moderate level of protection against challenge NDV replication as well as disease and death. Compared with APMV-9, the identity of F and HN proteins between APMV-3 and APMV-1 is 30.8% and 32.6%, respectively, but the level of protection by APMV-3 is more than that by APMV-9 (Nayak *et al.*, 2012).

2.11 APMV in Mammals

Only APMV-1 is known to naturally replicate in non-avian species, including humans. However, a virus of the APMV-2 serotype was isolated from cynomolgus monkeys, showing that the potential risk for infection may exist (Nishikawa *et al.*, 1977).

In humans, APMV-1 can cause an acute and rapidly clearing conjunctivitis, occasionally accompanied by low fever, headaches and chills. A single lethal case of pneumonia and systemic infection-associated virulent strain of APMV-1 was reported in an immunocompromised patient in the USA in 2007 (Goebel *et al.*, 2007). Human infections with APMV-1 have usually resulted from direct contact with infected birds at a farm or with the cultivated virus at a laboratory (Burnet, 1941; Quinn *et al.*, 1952; Capua and Alexander, 2004).

In the last 10 years several APMV-1 strains have been isolated from different outbreaks of fever and dyspnoea that occurred in pigs in China. Phylogenetic analysis revealed that many of these viruses are similar to the commonly utilized commercial La Sota and V4 vaccine strain in China (Ding *et al.*, 2010).

The potential use of NDV and other APMVs as a vaccine vector for the delivery of a foreign antigen not only in avians but also in other species including humans has been evaluated (Bukreyev and Collins, 2008). Until recently only PMV-1, the infection of APMV in non-avian species, has been investigated, but in a series of experiments at the University of Maryland, USA, Samuel and Khattar evaluated the replication and pathogenicity of APMVs in hamsters and mice (Khattar *et al.*, 2011; Samuel *et al.*, 2011). In hamsters, most of the APMV serotypes did not cause any clinical signs of disease; however, clinical signs of disease were observed only in the case of APMV-1 and APMV-9. All of the animals infected with APMV-9 exhibited clinical disease, including weakness and substantial weight loss. Virus was recovered 3 days post-infection from animals infected with APMV-1, -2, -3, -4, -6 and -9. The viral titres were moderate and were mostly restricted to the lungs and nasal turbinates; however, in a few cases viruses were isolated from the spleen (APMV-6), small intestine (APMV-6) or kidneys (APMV-4) of the infected animals (Samuel *et al.*, 2011). On the other hand, mice infected with the same serotypes exhibited loss of weight and a spectrum of mild, short-lived disease that was restricted to the respiratory tract when infected with APMV-1, -2, -6, -7 and -9. Except for APMV-5, each APMV induced a substantial humoral immune response in infected mice (Khattar *et al.*, 2011).

2.12 Laboratory Diagnosis

The main objectives in using the laboratory diagnosis of any infectious agents are to reach an adequate decision in managing the ongoing outbreak in order to implement disease control and prevention measures and to obtain evidence to support further epidemiological investigations. Disease investigation usually begins with the case history at the farm, including the description of the symptoms of the diseased animals. For proper investigation, a visit to the farm and examination of the flock is necessary. Animals showing typical signs of disease and freshly dead carcasses should be selected for autopsy either at the farm for field diagnosis or for examinations at the laboratory. Presumptive diagnosis can be made based on the history, described clinical symptoms and most prominent post-mortem findings. However, for definite diagnosis, laboratory examination,

including serological, virological and molecular tests, may be required, considering that many symptoms and pathological findings are not unique to one infection. Therefore, a suspicion of APMV infection cannot be conclusively diagnosed based on clinical signs and post-mortem findings. Many avian diseases like fowl cholera, infectious laryngotracheitis, infectious bronchitis and very virulent strains of infectious bursal disease show similar clinical signs. In addition, mismanagement at the animal farm, including food poisoning, could result in misdiagnosis of APMV infection.

2.12.1 Sampling

Intestinal contents, faeces, cloacal and tracheal/oropharyngeal swabs are shown to be most useful for the successful detection of APMV. Depending on the clinical signs, additional samples from the lung, spleen, liver, heart and brain should be considered when attempting isolation of the virus. In principle, all samples should be dealt with separately, but it is usual to make pools of the organs and tissues in practice. However, it is not recommended to pool the brain with any other tissue, especially for birds showing neurologic signs, or to pool tracheal and faecal tissues. Organs from different birds should not be pooled. All sample materials should be treated as potentially hazardous; thus handling the samples should strictly follow good laboratory practice. On arrival at the laboratory, samples should be processed as soon as possible. Putrefaction of the tissues and organs will surely affect the infectivity of the virus and therefore refrigerating the samples at 4°C during transport or storage should be considered. All of the material should be placed in sufficient antibiotic media. The type and concentration of the antibiotics used may vary depending on local conditions and the species of birds being examined. In general, a higher level of antibiotics should be used for cloacal swabs and faecal samples.

2.12.2 Serology

The usefulness of serological examinations in the diagnosis of APMVs is limited because of the widespread use of mass vaccination to control APMV-1 worldwide and the large number of serotypes represented among avian paramyxoviruses. However, the detection of immune response in unvaccinated birds may serve as a useful diagnostic function. In general, a higher level of antibody response could be observed following a more recent infection. Different serological techniques like enzyme-linked immunosorbent assay (ELISA) and the haemagglutination-inhibition (HI) test are routinely used in many laboratories to demonstrate antibodies against APMV-1. HI is the method recommended by the OIE and can be used for detecting antibodies against all avian paramyxoviruses except APMV-5, since the haemagglutination activity is not detected in all APMV-5 isolates. In unvaccinated birds, a positive HI response is confirmation of infection of the birds with that serotype. However, the possibility of cross-reactivity between different avian paramyxovirus serotypes should also be considered, especially between APMV-1, APMV-3 and APMV-7.

2.12.3 Virus isolation

Correct and rapid processing of the received samples enhances successful isolation of the virus. Avian paramyxoviruses will grow well in many different cell culture systems, but may require the addition of trypsin to facilitate growth in cell culture. The most widely used methods of cell culture are: chicken embryo liver, kidney and fibroblasts cells and African green monkey kidney (Vero) cells. However, the most sensitive method of isolation for all paramyxoviruses (except APMV-5), recommended and well-harmonized in the OIE diagnostic manual, is the use of embryonated chicken eggs. At least five embryonated chicken eggs, originating from specific pathogen-free (SPF) flocks, pre-incubated for 9–11 days at 37°C should be used in virus isolation attempts. Antibiotic-treated suspensions of organs

and tissues or swab washings are inoculated in the allantoic cavity of these embryonated chicken eggs. Inoculated eggs are incubated at 37°C for 5–7 days and candled daily. The amino allantoic fluids from dead or dying eggs as they arise and all eggs at the end of incubation period should be collected and tested for haemagglutination (HA) activity. For some avian paramyxoviruses, especially APMV-5, amniotic inoculation of 9 to 10-day-old SPF eggs and/or yolk sac inoculation of 6 to 7-day-old SPF eggs should also be considered in addition to the allantoic route. HA-negative allantoic fluid should be passaged repeating the abovementioned procedure two more times. HA-positive fluids need to be tested to exclude the presence of haemagglutinating bacteria. If the fluid is not contaminated with bacteria, haemagglutination activity will be due to either one of ten avian paramyxovirus serotypes or any avian influenza A virus subtypes. Serotype identification of the unknown isolate can be confirmed by using a standard HI test against a range of specific antiserum representing all serotypes of APMV and AI. After the initial isolation and identification of the virus, further characterization and laboratory assessments of the pathogenicity of the virus is necessary. Besides the molecular sequencing of the F gene cleavage site, three *in vivo* tests are used for this purpose: MDT in eggs, ICPI and intra-venous pathogenicity index (IVPI).

2.12.4 Detection of nucleic acid

Over the past decade, molecular techniques have become a recognized scientific tool for the detection and characterization of specific nucleic acid sequences. The highly sensitive techniques, like real-time reverse transcription polymerase chain reaction (rRT-PCR), random priming sequencing methods and next generation sequencing, enable the simultaneous detection, quantification, genotyping and genetic characterization of the virus directly from a wide range of clinical specimens and from different avian species. However, in the case of avian paramyxoviruses, as for many other RNA viruses, the evolutionary dynamics of the virus are driven by positive and negative selection pressures and the high inherent error rate of viral L (Holland *et al.*, 1982). Moreover, it has been suggested that recombination may also play a role in shaping the genetic diversity of avian paramyxoviruses (Miller *et al.*, 2009; Chong *et al.*, 2010). This rapid evolutionary dynamics plays a role in shaping the enormous genetic variation observed among avian paramyxoviruses and has a significant impact on diagnostics and disease control (Kim *et al.*, 2006; Cattoli *et al.*, 2010; Khan *et al.*, 2010; Rue *et al.*, 2010; Munir *et al.*, 2012b). Therefore, the implementation of molecular methods in the diagnosis of avian paramyxoviruses requires continuous epidemiological and molecular surveillance. Veterinary laboratories should employ more than one molecular technique targeting different regions of the genome in parallel, in order to avoid failure of detection. Various molecular approaches have been applied, mainly for diagnostic of APMV-1, in recent years (Aldous and Alexander, 2001) and these techniques have been reviewed recently in detail in several publications (Cattoli *et al.*, 2011; Hines and Miller, 2012). Use of several rRT-PCR assays has been reported from different laboratories to detect different genes of APMV-1 including the M, F and L (Wise *et al.*, 2004; Kim *et al.*, 2008; Fuller *et al.*, 2010; Hines *et al.*, 2012). Depending on the purpose, the molecular diagnosis of APMV-1 can be divided into two categories; (i) to detect APMV-1, highly-conserved targets like the M- and L-gene are chosen (Table 2.1) and (ii) to characterize and recognize virulence, a part of the F gene covering the region encoding the cleavage site is the target of different molecular tools. This molecular basis of virulence has been incorporated into the assessments of virulence in many laboratories. Recently, a novel real-time PCR-based strategy has been developed for simple and rapid molecular pathotyping of APMV-1 (Yacoub *et al.*, 2012). This strategy is based on the sequence at the cleavage site, which reflects the pathogenicity of the APMV-1. The presence of multiple basic amino acids at the F0

Table 2.1. Published real-time PCR assays for the detection of NDV genomes.

Genomic target	Primer/probe name	Sequence of the primer 5′ to 3′	Reference
M gene	M+4100	GTGATGTGCTCGGACCTTC	Wise *et al.* (2004)
	M+6169	*[FAM]*TTCTCTAGCAGTGGGACAGCCTGC*[TAMRA]*	
	M-4220	CCTGAGGAGAGGCATTTGCTA	
F gene	F+4839	TCCGGAGGATACAAGGGTCT	Wise *et al.* (2004)
	F+4894	*[FAM]*AAGCGTTTCTGTCTCCTTCCTCCA*[TAMRA]*	
	F-4939	AGCTGTTGCAACCCCAAG	
L gene	ND-F	GAGCTAATGAACATTCTTTC	Fuller *et al.* (2010)
	ND-R	AATAGGCGGACCACATCTG	
	NDpro1	*[FAM]*TCATTCTTTATAGAGGTATCTTCATCATA*[BHQ1]*	
	NDpro2	*[FAM]*TCATACACTATTATGGCGTCATTCTT*[BHQ1]*	

cleavage site (^{113}RQK/RR*F^{117}) in combination with a basic amino acid at position 112 and phenylalanine at residues 117 has been shown to be directly related to the virulence of viruses *in vivo*. In contrast, viruses of low virulence usually have the sequence (^{113}RQK/RR*F^{117}).

Although improvements have been made to PCR protocols for the detection of APMV-2–10 viruses over the past decade, the availability of rapid molecular techniques for the detection of these viruses is still very limited. This is mainly because of the limited availability of sequence information for these serotypes and the lack of studies investigating the genetic variability within circulating populations of avian paramyxoviruses. Therefore, there is still a need for more reliable molecular assays for the detection and identification of strains of avian paramyxoviruses.

References

Aldous, E.W. and Alexander, D.J. (2001) Detection and differentiation of Newcastle disease virus (avian paramyxovirus type 1). *Avian Pathology* 30, 117–128.

Aldous, E.W., Mynn, J.K., Banks, J. and Alexander, D.J. (2003) A molecular epidemiological study of avian paramyxovirus type 1 (Newcastle disease virus) isolates by phylogenetic analysis of a partial nucleotide sequence of the fusion protein gene. *Avian Pathology* 32, 239–256.

Aldous, E.W., Fuller, C.M., Mynn, J.K. and Alexander, D.J. (2004) A molecular epidemiological investigation of isolates of the variant avian paramyxovirus type 1 virus (PPMV-1) responsible for the 1978 to present panzootic in pigeons. *Avian Pathology* 33, 258–269.

Alexander, D.J. (1980) Avian paramyxoviruses. *Veterinary Bulletin* 50, 737–752.

Alexander, D.J. (2003) Avian paramyxoviruses 2–9. In: Saif, Y.M. (ed.) *Disease of Poultry*. Iowa State University Press, Ames, Iowa, pp. 88–92.

Alexander, D.J. (2011) Newcastle disease in the European Union 2000 to 2009. *Avian Pathology* 40, 547–558.

Alexander, D.J. and Chettle, N.J. (1978) Relationship of parakeet/Netherland/449/75 virus to other avian paramyxoviruses. *Research in Veterinary Science* 25, 105–106.

Alexander, D.J. and Collins, M.S. (1982) Pathogenecity of PMV-3/parakeet/Netherland/449/75 for chickens. *Avian Pathology* 11, 179–185.

Alexander, D.J., Chettle, N.J. and Parsons, G.P. (1979a) Resistance of chickens to challenge with the virulent Hertz 33 strain of Newcastle disease virus induced by prior infection with serologically distinct avian paramyxoviruses. *Research in Veterinary Science* 26, 198–201.

Alexander, D.J., Aymard, M., Kessler, N. and Collins, M.S. (1979b) Antigenic and structural relationships between avian paramyxoviruses isolated from ducks in Hong Kong and Mississippi, USA. *Journal of General Virology* 44, 839–842.

Alexander, D.J., Hinshaw, V.S. and Collins, M.S. (1981) Characterization of viruses from doves representing a new serotype of avian paramyxoviruses. *Archives of Virology* 68, 265–269.

Alexander, D.J., Russell, P.H. and Collins, M.S. (1984) Paramyxovirus type 1 infections of racing pigeons: 1. Characterisation of isolated viruses. *Veterinary Record* 114, 444–446.

Alexander, D.J., Manvell, R.J., Collins, M.S. and Bockman, S.J. (1991) Evaluation of relationship between avianparamxoviruses isolated from birds of the *Columbidae* family. *Archives of Virology* 114, 267–276.

Bankowski, R.A. and Corstvetre, C.G. (1960) Isolation of an unidentified agent from the respiratory tract of chickens. *Science* 29, 292–293.

Bankowski, R.A., Conrad, R.D. and Reinolds, B. (1968) Avian influenza A and paramyxoviruses complicating respiratory disease diagnosis in poultry. *Avian Diseases* 12, 259–278.

Bankowski, R.A., Almquist, J. and Dombrucki, J. (1981) Effect of paramyxovirus yucaipa on fertility, hatchability, and poult yield of turkeys. *Avian Diseases* 25, 517–520.

Beck, I., Gerlach, H., Burkhardt, E. and Kaleta, E.F. (2003) Investigation of several selected adjuvants regarding their efficacy and side effects for the production of a vaccine for parakeets to prevent a disease caused by a paramyxovirus type 3. *Vaccine* 21, 1006–1022.

Bukreyev, A. and Collins, P.L. (2008) Newcastle disease virus as a vaccine vector for humans. *Current Opinion in Molecular Therapeutics* 10, 46–55.

Burnet, F.M. (1943) Human infection with the virus of Newcastle disease of fowls. *Medical Journal of Australia* 2, 313–314.

Capua, I. and Alexander, D.J. (2004) Human health implications of avian influenza viruses and paramyxoviruses. *European Journal of Clinical Microbiology & Infectious Diseases* 23, 1–6.

Capua, I., Scacchia, M., Toscani, T. and Caporale, V. (1993) Unexpected isolation of virulent Newcastle disease virus from commercial embryonated fowls' eggs. *Journal of Veterinary Medicine B* 40, 609–612.

Capua, I., De Nardi, R., Beato, M.S., Terregino, C., Scremin, M. and Guberti, V. (2004) Isolation of an avian paramyxovirus type 9 from migratory waterfowl in Italy. *Veterinary Record* 155, 156.

Cattoli, G., Fusaro, A., Monne, I., Molia, S., Le Menach, A., Maregeya, B., Nchare, A., Bangana, I., Maina, A.G., Koffi, J.N., Thiam, H., Bezeid, O.E., Salviato, A., Nisi, R., Terregino, C. and Capua, I. (2010) Emergence of a new genetic lineage of Newcastle disease virus in West and Central Africa implications for diagnosis and control. *Veterinary Microbiology* 142, 168–176.

Cattoli, G., Susta, L., Terregino, C. and Brown, C. (2011) Newcastle disease: a review of field recognition and current methods of laboratory detection. *Journal of Veterinary Diagnostic Investigations* 23, 637–656.

Chang, P.C., Hsieh, M.L., Shien, J.H., Graham, D.A., Lee, M.S. and Shiehm, H. (2001) Complete nucleotide sequence of avian paramyxovirus type 6 isolated from ducks. *Journal of General Virology* 82, 2157–2168.

Cheville, N.F., Stone, H., Riley, J. and Ritchie, A.E. (1972) Pathogenesis of virulent Newcastle disease in chickens. *Journal of the American Veterinary Medical Association* 161, 169–179.

Chong, Y.L., Padhi, A., Hudson, P.J. and Poss, M. (2010) The effect of vaccination on the evolution and population dynamics of Avian paramyxovirus-1. *PLoS Pathogen* 6, e1000872.

Cloud, S.S. and Rosenberger, J.K. (1980) Characterization of nine avian paramyxoviruses. *Avian Diseases* 24, 139–152.

Collins, M.S., Alexander, D.J., Brockman, S., Kemp, P.A. and Manvell, R.J. (1989) Evaluation of mouse monoclonal antibodies raised against an isolate of the variant avian paramyxovirus type 1 responsible for the current panzootic in pigeons. *Archives of Virology* 104, 53–61.

Czegledi, A., Herczeg, J., Hadjiev, G., Doumanova, L., Wehmann, E. and Lomniczi, B. (2002) The occurrence of five major Newcastle disease virus genotypes (II, IV, V, VI and VIIb) in Bulgaria between 1959 and 1996. *Epidemiology and Infection* 129, 679–688.

Ding, Z., Cong, Y.l., Chang, S., Wang, G.M., Wang, Z., Zhang, Q.P., Wu, H. and Sun, Y.Z. (2010) Genetic analysis of avian paramyxovirus-1 (Newcastle disease virus) isolates obtained from swine populations in China related to commonly utilized commercial vaccine strains. *Virus Genes* 41, 369–376.

Dortmans, J.C., Rottier, P.J., Koch, G. and Peeters, B.P. (2011) Passaging of a Newcastle disease virus pigeon variant in chickens results in selection of viruses with mutations in the polymerase complex enhancing virus replication and virulence. *Journal of General Virology* 92, 336–345.

Doyle, T.M. (1927) A hitherto unrecorded disease of fowls due to a filter-passing virus. *Journal of Comparative Pathology and Therapeutics* 40, 144–169.

Erickson, G.A., Brugh, M. and Beard, C.W. (1980) Viscerotropic velogenic Newcastle disease in pigeons: clinical disease and immunization. *Avian Diseases* 24, 257–267.

Fleury, H.J. and Alexander, D.J. (1979) Isolation of twenty-three Yucaipa-like viruses from 616 wild birds in Senegal, West Africa. *Avian Diseases* 23, 742–744.

Fornells, L.A., Silva, T.F., Bianchi, I., Travassos, C.E., Liberal, M.H., Andrade, C.M., Petrucci, M.P., Veiga, V.F., Vaslin, M.F. and Couceiro, J.N. (2012) Detection of paramyxoviruses in Magellanic penguins (*Spheniscus magellanicus*) on the Brazilian tropical coast. *Veterinary Microbiology* 4, 429–433.

Friend, M. and Trainer, D.O. (1972) Experimental Newcastle disease studies in the mallard. *Avian Diseases* 16, 700–713.

Fuller, C.M., Brodd, L., Irvine, R.M., Alexander, D.J. and Aldous, E.W. (2010) Development of an L gene real-time reverse-transcription PCR assay for the detection of avian paramyxovirus type 1 RNA in clinical samples. *Archives of Virology* 155, 817–823.

Goebel, S.J., Taylor, J., Barr, B.C., Kiehn, T.E., Castro-Malaspina, H.R., Hedvat, C.V., Rush-Wilson, K.A., Kelly, C.D., Davis, S.W., Samsonoff, W.A., Hurst, K.R., Behr, M.J. and Masters, P.S. (2007) Isolation of avian paramyxovirus 1 from a patient with a lethal case of pneumonia. *Journal of Virology* 81, 12709–12714.

Goodman, B.B. and Hanson, R.P. (1988) Isolation of avian paramyxovirus-2 from domestic and wild birds in Costa Rica. *Avian Diseases* 32, 713–717.

Gorman, J.J., Nestorowicz, A., Mitchell, S.J., Corino, G.L. and Selleck, P.W. (1988) Characterization of the sites of proteolytic activation of Newcastle disease virus membrane glycoprotein precursors. *The Journal of Biological Chemistry* 263, 12522–12531.

Gough, R.E. and Alexander, D.J. (1984) Avian paramyxovirus type 4 isolated from a ringed teal (*Calonetta leucophrys*). *Veterinary Record* 115, 653.

Gough, R.E., Manvell, R.J., Drury, S.E., Naylor, P.F., Spackman, D. and Cooke, S.W. (1993) Deaths in budgerigars associated with a paramyxovirus-like agent. *Veterinary Record* 133, 123.

Haller, O., Kochs, G. and Weber, F. (2006) The interferon response circuit: induction and suppression by pathogenic viruses. *Virology* 344, 119–130.

Hanson, R.P. and Sinha, S.K. (1951) Epizootic of Newcastle disease in pigeons and studies on transmission of the virus. *Poultry Science* 31, 404–408.

Hines, N.L. and Miller, C.L. (2012) Avian paramyxovirus serotype-1: a review of disease distribution, clinical symptoms, and laboratory diagnostics. *Veterinary Medicine International* Article ID 708216, doi:10.1155/2012/708216.

Hines, N.L., Killian, M.L. and Pedersen, J.C. (2012) An rRT-PCR assay to detect the matrix gene of a broad range of avian paramyxovirus serotype-1 strains. *Avian Diseases* 56, 387–395.

Holland, J., Spindler, K., Horodyski, F., Grabau, E., Nichol, S. and Vande, P.S. (1982) Rapid evolution of RNA genomes. *Science* 215, 1577–1585.

Huang, Z., Krishnamurthy, S., Panda, A. and Samal, S.K. (2003) Newcastle disease virus V protein is associated with viral pathogenesis and functions as an alpha interferon antagonist. *Journal of Virology* 77, 8676–8685.

Huang, Z., Panda, A., Elankumaran, S., Govindarajan, D., Rockemann, D.D. and Samal, S.K. (2004) The hemagglutinin-neuraminidase protein of Newcastle disease virus determines tropism and virulence. *Journal of Virology* 78, 4176–4184.

Iyer, S.G. (1939) Paralysis in a pigeon due to Doyle's (Ranikhet) disease virus. *Indian Journal of Veterinary Science and Animal Husbandry* 9, 379–382.

Jeon, W.J., Lee, E.K., Kwon, J.H. and Choi, K.S. (2008) Full-length genome sequence of avian paramyxovirus type 4 isolated from a mallard duck. *Virus Genes*, 37, 342–350.

Jestin, V., Cherbonnel, M., Morin, M., Guittet, M. and Bennejean, G. (1989) Characterization of French avian paramyxovirus type 1 (PMV 1) isolates with a panel of monoclonal antibodies to the Ploufragan strain of Newcastle disease virus. *Archives of Virology* 105, 189–198.

Jinding, C., Ming, L., Tao, R. and Chaoan, X. (2005) A goose-sourced paramyxovirus isolated from Southern China. *Avian Diseases* 49, 170–173.

Kaleta, E.F. and Baldauf, C. (1988) Newcastle disease in free-living and pet birds. In: Alexander, J.D. (ed.) *Newcastle Disease*. Kluwer Academic, Boston, Massachusetts, pp. 197–246.

Kaleta, E.F., Alexander, D.J., Russell, P.H. (1985) The first isolation of the PMV-1 virus responsible for the current panzootic in pigeons. *Avian Pathology* 14, 553–557.

Kaleta, E.F., Werner, O. and Hemberger, Y. (2010) Isolation and characterization of avian paramyxovirus type 3b from farmed Namibian ostriches (*Struthio camelus*). *Berl Munch Tierarztl Wochenschr* 123, 103–110.

Kaschula, V.R. (1952) The domestic pigeon as possible carrier of Newcastle disease. *Ondersteport Journal of Veterinary Research* 25, 25–27.

Khan, A., Rue, C.A., Rehmani, S.F., Ahmed, A., Wasilenko, J.L., Miller, P.J. and Afonso, C.L. (2010) Phylogenetic and biological characterization of Newcastle disease virus isolates from Pakistan. *Journal of Clinical Microbiology* 48, 1892–1894.

Khattar, S.K., Kumar, S., Xiao, S., Collins, P.L. and Samal, S.K. (2011) Experimental infection of mice with avian paramyxovirus serotypes 1 to 9. *PLoS One* 6, e16776.

Kim, L.M., Afonso, C.L. and Suarez, D.L. (2006) Effect of probe-site mismatches on detection of virulent Newcastle disease viruses using a fusion-gene real-time reverse transcription polymerase chain reaction test. *Journal of Veterinary Diagnostic Investigation* 18, 519–528.

Kim, L.M., Suarez, D.L. and Afonso, C.L. (2008) Detection of a broad range of class I and II Newcastle disease viruses using a multiplex real-time reverse transcription polymerase chain reaction assay. *Journal of Veterinary Diagnostic Investigation* 20, 414–425.

Kim, S.H, Xiao, S., Shive, H., Collins, P.L. and Samal, S.K. (2012) Replication, neurotropism, and pathogenicity of avian paramyxovirus serotypes 1–9 in chickens and ducks. *PLoS One* 7, e34927.

Kolakofsky, D., Roux, L., Garcin, D. and Ruigrok, R.W. (2005) Paramyxovirus mRNA editing, the 'rule of six' and error catastrophe: a hypothesis. *Journal of General Virology* 86, 1869–1877.

Krishnamurthy, S. and Samal, S.K. (1998) Nucleotide sequences of the trailer, nucleocapsid protein gene and intergenic regions of Newcastle disease virus strain Beaudette C and completion of the entire genome sequence. *Journal of General Virology* 79, 2419–2424.

Kumar, S., Nayak, B., Collins, P.L. and Samal, S.K. (2008) Complete genome sequence of avian paramyxovirus type 3 reveals an unusually long trailer region. *Virus Research* 137, 189–197.

Kumar, S., Nayak, B., Samuel, A.S., Xiao, S., Collins, P.L. and Samal, S.K. (2010) Complete genome sequence of avian paramyxovirus-3 strain Wisconsin: evidence for the existence of subgroups within the serotype. *Virus Research* 149, 78–85.

Lamb, R.A. and Kolakofsky, D. (2001) *Paramyxoviridae*: the viruses and their replication. In: Knipe, D.M. and Howley, P.M. (eds) *Fundamental Virology*, 4th edn. Lippincott, Williams & Wilkins, Philadelphia, Pennsylvania, pp. 689–724.

Lamb, R.A., Collins, P.L., Kolakofsky, D., Melero, J.A., Nagai, Y., Oldstone, M.B.A., Pringle, C.R. and Rima, B.K. (2005) Family *paramyxoviridae*. In: Fauquet, C.M. (ed.) *Virus Taxonomy: The Classification and Nomenclature of Viruses. The Eighth Report of the International Committee in Taxonomy of Viruses*. ICTV.

Lamb, R.A., Paterson, R.G. and Jardetzky, T.S. (2006) Paramyxovirus membrane fusion: lessons from the F and HN atomic structures. *Virology* 344, 30–37.

Leeuw, O.S. de, Koch, G., Hartog, L., Ravenshorst, N. and Peeters, B.P.H. (2005) Virulence of Newcastle disease virus is determined by the cleavage site of the fusion protein and by both the stem region and globular head of the haemagglutinin-neuraminidase protein. *Journal of General Virology* 86, 1759–1769.

Lipkind, M. and Shihmanter, E. (1986) Antigenic relationships between avian paramyxoviruses. I. Quantitative characteristics based on hemagglutination and neuraminidase inhibition tests. *Archives of Virology* 89, 89–111.

Liu, X.F., Wan, H.Q., Ni, X.X., Wu, Y.T. and Liu, W.B. (2003) Pathotypical and genotypical characterization of strains of Newcastle disease virus isolated from outbreaks in chicken and goose flocks in some regions of China during 1985–2001. *Archives of Virology* 148, 1387–1403.

Lomniczi, B., Wehmann, E., Herczeg, J., Ballagi-Pordany, A., Kaleta, E.F., Werner, O., Meulemans, G., Jorgensen, P.H., Mante, A.P., Gielkens, A.L., Capua, I. and Damoser, J. (1998) Newcastle disease outbreaks in recent years in Western Europe were caused by an old (VI) and a novel genotype (VII). *Archives of Virology* 143, 49–64.

Macpherson, I., Watt, R.G. and Alexander, D.J. (1983) Isolation of avian paramyxovirus other than Newcastle disease virus from commercial poultry in Great Britain. *Veterinary Record* 112, 479–480.

Maldonado, A., Arenas, A., Tarradas, M.C., Luque, I., Astorga, R., Perea, J.A. and Miranda, A. (1995) Serological survey for avian paramyxoviruses from wildfowl in aquatic habitats in Andalusia. *Journal of Wildlife Diseases* 31, 66–69.

Marius-Jestin, V., Cherbonnel, M., Picault, J.P. and Bennejean, G. (1987) Isolation from ducks of a hyper-virulent strain of duck plague virus and an avian type 6 paramyxovirus. *Comparative Immunology, Microbiology and Infectious Disease* 10, 173–186.

McFerran, J.B., Connor, T.J., Allan, G.M., Purcell, D.A.P. and Young, J.A. (1973) Studies on a virus isolated from a finch. *Research in Veterinary Science* 15, 116–118.

Mebatsion, T., Verstegen, S., De Vaan, L.T., Romer-Oberdorfer, A. and Schrier, C.C. (2001) A recombinant Newcastle disease virus with low-level V protein expression is immunogenic and lacks pathogenicity for chicken embryos. *Journal of Virology* 75, 420–428.

Meteyer, C.U., Docherty, D.E., Glaser, L.C., Franson, J.C.F., Senne, D.A. and Duncan, R. (1997) Diagnostic findings in the 1992 epornitic of neurotropic velogenic Newcastle disease in double-crested cormorants from the upper midwestern United States. *Avian Diseases* 41, 171–180.

Miller, P.J., Kim, L.M., Ip, H.S. and Afonso, C.L. (2009) Evolutionary dynamics of Newcastle disease virus. *Virology* 391, 64–72.

Miller, P.J., Afonso, C.L., Spackman, E., Scott, M.A., Pedersen, J.C., Senne, D.A., Brown, J.D., Fuller, C.M., Uhart, M.M., Karesh, W.B., Brown, I.H., Alexander, D.J. and Swayne, D.E. (2010a) Evidence for a new avian paramyxovirus serotype 10 detected in rockhopper penguins from the Falkland Islands. *Journal of Virology* 84, 11,496–11,504.

Miller, P.J., Decanini, E.L. and Afonso, C.L. (2010b) Newcastle disease: evolution of genotypes and the related diagnostic challenges. *Infection, Genetics and Evolution* 10, 26–35.

Munir, M., Zohari, S. and Berg, M. (2012a) Newcastle disease virus in Pakistan: genetic characterization and implication in molecular diagnosis. *Indian Journal of Virology* 23, 368–373.

Munir, M., Cortey, M., Abbas, M., Qureshi, Z.U., Afzal, F., Shabbir, M.Z., Khan, M.T., Ahmed, S., Ahmad, S., Baule, C., Ståhl, K., Zohari, S. and Berg, M. (2012b) Biological characterization and phylogenetic analysis of a novel genetic group of Newcastle disease virus isolated from outbreaks in commercial poultry and from backyard poultry flocks in Pakistan. *Infection Genetics and Evolution* 12, 1010–1019.

Mustaffa-Babjee, A., Spradbrow, P.B. and Samuel, J.L. (1974) A pathogenic paramyxovirus from a budgerigar (*Melopsittacus undulatus*). *Avian Diseases* 18, 226–230.

Nakaya, T., Cros, J., Park, M.S., Nakaya, Y., Zheng, H., Sagrera, A., Villar, E., García-Sastre, A. and Palese, P. (2001) Recombinant Newcastle disease virus as a vaccine vector. *Journal of Virology* 75, 11,868–11,873.

Nayak, B., Kumar, S., Collins, P.L. and Samal, S.K. (2008) Molecular characterization and complete genome sequence of avian paramyxovirus type 4 prototype strain duck/Hong Kong/D3/75. *Virology Journal* 5, 124.

Nayak, B., Dias, F.M., Kumar, S., Paldurai, A., Collins, P.L. and Samal, S.K. (2012) Avian paramyxovirus serotypes 2–9 (APMV-2-9) vary in the ability to induce protective immunity in chickens against challenge with virulent Newcastle disease virus (APMV-1). *Vaccine* 30, 2220–2227.

Nerome, K., Nakayama, M., Ishida, M. and Fukumi, H. (1978) Isolation of a new avian paramyxovirus from budgerigar (*Melopsittacus undulatus*). *Journal of General Virology* 38, 293–301.

Nishikawa, F., Sugiyama, T. and Suzuki, K. (1977) A new paramyxovirus isolated from cynomolgus monkeys. *Japanese Journal of Medical Science & Biology* 30, 191–204.

OIE (2012) Office International des Epizooties (OIE-World Organization for Animal Health) http://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2012.

Paldurai, A., Subbiah, M., Kumar, S., Collins, P.L. and Samal, S.K. (2009) Complete genome sequences of avian paramyxovirus type 8 strains goose/Delaware/1053/76 and pintail/Wakuya/20/78. *Virus Research* 142, 144–153.

Park, M.S., Garcia-Sastre, A., Cros, J.F., Basler, C.F. and Palese. P. (2003) Newcastle disease virus V protein is a determinant of host range restriction. *Journal of Virology* 77, 9522–9532.

Quinn, R.W., Hanson, R.P., Brown, J.W. and Brandly, C.A. (1952) Newcastle disease virus in man. Results of studies in five cases. *Journal of Laboratory and Clinical Medicine* 40, 736–743.

Redmann, T., Zeydanli, M.M., Herbst, W. and Kaleta, E.F. (1991) Isolation of a paramyxovirus-3 from turkeys with respiratory tract disease in Germany. *Deutsche Tieraertliche Wochenschrift* 98, 138–141.

Rosseel, T., Lambrecht, B., Vandenbussche, F., Van den Berg, T. and Van Borm, S. (2011) Identification and complete genome sequencing of paramyxoviruses in mallard ducks (*Anas platyrhynchos*) using random access amplification and next generation sequencing technologies. *Virology Journal* 8, 463.

Rue, C.A., Susta, L., Brown, C.C., Pasick, J.M., Swafford, S.R., Wolf, P.C., Killian, M.L., Pedersen, J.C., Miller, P.J. and Afonso, C.L. (2010) Evolutionary changes affecting rapid identification of 2008 Newcastle disease viruses isolated from double-crested cormorant. *Journal of Clinical Microbiology* 48, 2440–2448.

Saif, Y.M., Mohan, R., Ward, L., Senne, D.A., Panigrahy, B. and Dearth, R.N. (1997) Natural and experimental infection of turkeys with avian paramyxovirus-7. *Avian Diseases* 41, 326–329.

Samuel, A.S., Kumar, S., Madhuri, S., Collins, P.L. and Samal, S.K. (2009) Complete sequence of the genome of avian paramyxovirus type 9 and comparison with other paramyxoviruses. *Virus Research* 142, 10–18.

Samuel, A.S., Paldurai, A., Kumar, S., Collins, P.L. and Samal, S.K. (2010) Complete genome sequence of avian paramyxovirus (APMV) serotype 5 completes the analysis of nine APMV serotypes and reveals the longest APMV genome. *PLoS ONE* 5, e9269.

Samuel, A.S., Subbiah, M., Shive, H., Collins, P.L. and Samal, S.K. (2011) Experimental infection of hamster with avian paramyxovirus serotype 1 to 9. *Veterinary Research* 48, 39.

Samuel, C.E. (2001) Antiviral actions of interferons. *Clinical Microbiology Reviews* 14, 778–809.

Sandhu, T. and Hinshaw, V. (1981) Influenza A virus infection of domestic ducks. *First International Symposium of Avian Influenza* 93–99.

Senne, D.A., Pearson, J.E., Miller, L.D. and Gustafson, G.A. (1983) Virus isolations from pet birds submitted for importation into the United States. *Avian Diseases* 27, 731–744.

Shortridge, K.F. and Alexander, D.J. (1978) Incidence and preliminary characterisation of a hitherto unreported, serologically distinct, avian paramyxovirus isolated in Hong Kong. *Research in Veterinary Science* 25, 128–130.

Shortridge, K.F., Alexander, D.J. and Collins, M.S. (1980) Isolation and properties of viruses from poultry in Hong Kong which represent a new (sixth) distinct group of avian paramyxoviruses. *Journal of General Virology* 49, 255–262.

Stanislawek, W.L., Wilks, C.R., Meers, J., Horner, G.W., Alexander, D.J., Manvell, R.J., Kattenbelt, J.A. and Gould, A.R. (2002) Avian paramyxoviruses and influenza viruses isolated from mallard ducks (*Anas platyrhynchos*) in New Zealand. *Archives of Virology* 147, 1287–1302.

Steward, M., Vipond, I., Millar, N.S. and Emmerson, P.T. (1993) RNA editing in Newcastle disease virus. *Journal of General Virology* 74, 2539–2547.

Stewart, G.H. (1971) Naturally occurring clinical Newcastle disease in the racing pigeon (*Columba livia*). *Veterinary Record* 89, 225–226.

Subbiah, M., Xiao, S., Collins, P.L. and Samal, S.K. (2008) Complete sequence of the genome of avian paramyxovirus type 2 (strain Yucaipa) and comparison with other paramyxoviruses. *Virus Research* 137, 40–48.

Subbiah, M., Nayaka, S., Collins, P.L., Siba, K. and Samal, S.K. (2010) Complete genome sequences of avian paramyxovirus serotype 2 (APMV-2) strains Bangor, England and Kenya: evidence for the existence of subgroups within serotype 2. *Virus Research* 152, 85–95.

Tantawi, H.H., Al Falluji, M.M. and Al Sheikhly, F. (1979) Viral encephalomyelitis of pigeons: identification and characterization of the virus. *Avian Diseases* 23, 785–793.

Tumova, B., Robinson, J.H. and Easterday, B.C. (1979a) A hitherto unreported paramyxovirus of turkey. *Research in Veterinary Science* 27, 135–140.

Tumova, B., Stumpa, A., Janout, V., Uvizl, M. and Chmela, J. (1979b) A further member of the Yucaipa group isolated from the common wren (*Troglodytes troglodytes*). *Archives of Virology* 23, 504–507.

Tumova, B., Bachmann, P.A., Eichhorn, W., Pfleger, A. and Stumpa, A. (1989) Further evidence of the circulation of PMV-4 and influenza viruses with N2-1957 enzyme in the migratory waterfowls. *Acta Virologica* 33, 573–576.

Vindevogel, H., Thiry, E., Pastoret, P.P. and Meulemans, G. (1982) Lentogenic strains of Newcastle disease virus in pigeons. *Veterinary Record* 110, 497–499.

Wan, H., Chen, L., Wu, L. and Liu, X. (2004) Newcastle disease in geese: natural occurrence and experimental infection. *Avian Pathology* 33, 216–221.

Warke, A., Stallknecht, D., Williams, S.M., Pritchard, N. and Mundt, E. (2008) Comparative study on the pathogenicity and immunogenicity of wild bird isolates of avian paramyxovirus 2, 4, and 6 in chickens. *Avian Pathology* 37, 429–434.

Webster, R.G., Morita, M., Pridgen, C. and Tumova, B. (1976) Orthoand paramyxoviruses from migrating feral ducks: characterization of a new group of influenza A viruses. *Journal of General Virology* 32, 217–225.

Wilson, R.L., Fuentes, S.M., Wang, P., Taddeo, E.C., Klatt, A., Henderson, A.J. and He, B. (2006) Function of small hydrophobic proteins of paramyxovirus. *Journal of Virology* 80, 1700–1709.

Wise, M.G., Suarez, D.L., Seal, B.S., Pedersen, J.C., Senne, D.A., King, D.J., Kapczynski, D.R. and Spackman, E. (2004) Development of a real-time reverse-transcription PCR for detection of Newcastle disease virus RNA in clinical sample. *Journal of Clinical Microbiology* 42, 329–338.

Wobeser, G.F., Leighton, F.A., Norman, R., Meyers, D.J., Onderka, D., Pybus, M.J., Neufeld, J.L., Fox, G.A. and Alexander, D.J. (1993) Newcastle disease in wild water birds in western Canada, 1990. *Canada Veterinary Journal* 34, 353–359.

Wood, A.M., Dagless, M.D., Pirie, J.O., Garcia-Rueda, M.C., Manvell, R.J., Cox, W.J., Ceeraz, V., Pearson, D.B., Law, W.A., Alexander, D.J. and Brown, I.H. (2008) Isolations of avian paramyxovirus type 2 from domestic fowl in Scotland in 2002 and 2006. *Veterinary Record* 162, 788–789.

Woolcock, P.R., Moore, J.D., McFarland, M.D. and Panigrahy, B. (1996) Isolation of paramyxovirus serotype 7 from ostriches (*Struthio camelus*). *Avian Diseases* 40, 945–949.

Xiao, S., Paldural, A., Nayak, B., Subbiah, M., Collins, P.L. and Samal, S.K. (2009) Complete genome sequence of avian paramyxovirus type 7 (strain Tennessee) and comparison with other paramyxoviruses. *Virus Research* 145, 80–91.

Xiao, S., Subbiah, M., Kumar, S., De Nardi, R., Terregino, C., Collins, P.L. and Samal, S.K. (2010) Complete genome sequences of avian paramyxovirus serotype 6 prototype strain Hong Kong and a recent novel strain from Italy: evidence for the existence of subgroups within the serotype. *Virus Research* 150, 61–72.

Yacoub, A., Leijon, M., McMenamy, M.J., Ullman, K., McKillen, J., Allan, G. and Belák, S. (2012) Development of a novel real-time PCR-based strategy for simple and rapid molecular pathotyping of Newcastle disease virus. *Archives of Virology* 157, 833–844.

Yamane, N., Arikawa, J., Odagiri, T. and Ishida, N. (1982) Characterization of avian paramyxoviruses isolated from feral ducks in northern Japan: the presence of three distinct viruses in nature. *Microbiology and Immunology* 26, 557–568.

Zhang, G.Z., Zhao, J.X., Wang, H.W., Yang, A.M., Bu, C.Y. and Wang, M. (2006) Isolation, identification, and comparison of four isolates of avian paramyxovirus serotype 2 in China. *Avian Diseases* 50, 386–390.

Zhang, G.Z., Zhao, J.X. and Wang, M. (2007) Serological survey on prevalence of antibodies to avian paramyxovirus serotype 2 in China. *Avian Diseases* 51, 137–139.

3 Hendra and Nipah Viruses

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3.1 Introduction

Hendra (HeV) and Nipah (NiV) viruses are two of the most dangerous viruses in the world. Their emergence from a zoonotic reservoir in South-east Asia and Oceania caused human outbreaks with fatality rates rising up to 100% among infected people. Due to their high pathogenicity and the lack of appropriate prophylaxis or treatment, HeV and NiV are classified as biosafety level 4 (BSL-4) pathogens. They are also classified as category C potential agents of bioterrorism. The study of these intriguing viruses is therefore particularly restricted and hampered for security reasons.

In 1994, a new virus causing severe respiratory disease in horses was identified in the Brisbane suburb of Hendra, Australia. The Hendra virus was rapidly proved to cause severe illness in humans who had been in close contact with infected horses (Murray *et al.*, 1995). In 1998, in Malaysia, a new disease emerged in pigs, causing large epizootics that were initially thought to be due to Japanese encephalitis virus (JeV). Because many of the people exposed to the porcine epizootic tested negative for JeV, the emergence of a new virus was suggested. In 1999, the Nipah virus was first isolated from a human fatality from the Sungai Nipah village (Mohd Nor *et al.*, 2000). These viruses show features of the *Paramyxoviridae* family but possess particularities that led to the creation of the *Henipavirus* genus, named after the contraction of the two names. Henipaviruses are large-enveloped single-stranded RNA viruses of negative polarity (Fig. 3.1). Their genome is the largest among the *Paramyxoviridae* viruses (≈18.2 kb) and contains six transcriptional units coding for six structural proteins (N, P, M, F, G and F). Within the phosphoprotein (P) gene, the presence of a single editing site allows for the addition of one or two Guanine (G) residues during transcription by the viral polymerase. Translation of edited transcripts results in proteins that share the same amino-terminus as that of P protein but each carries a unique carboxyl-terminus. The protein with the +1 frameshift corresponds to the V protein and the protein with the +2 frameshift is known as the W protein. Another open reading frame within the P gene allows the production of a small protein known as C protein (Eaton *et al.*, 2006). The P, V, W and C proteins were proved to be involved in the inhibition of the immune host response and act as virulence factors (Basler, 2012).

HeV and NiV are both harboured by several species of fruit bats from the *Pteropodidae* family, which range from South-east Asia and Australia to Africa, and better known as flying foxes (Fig. 3.2). These giant bats, displaying a

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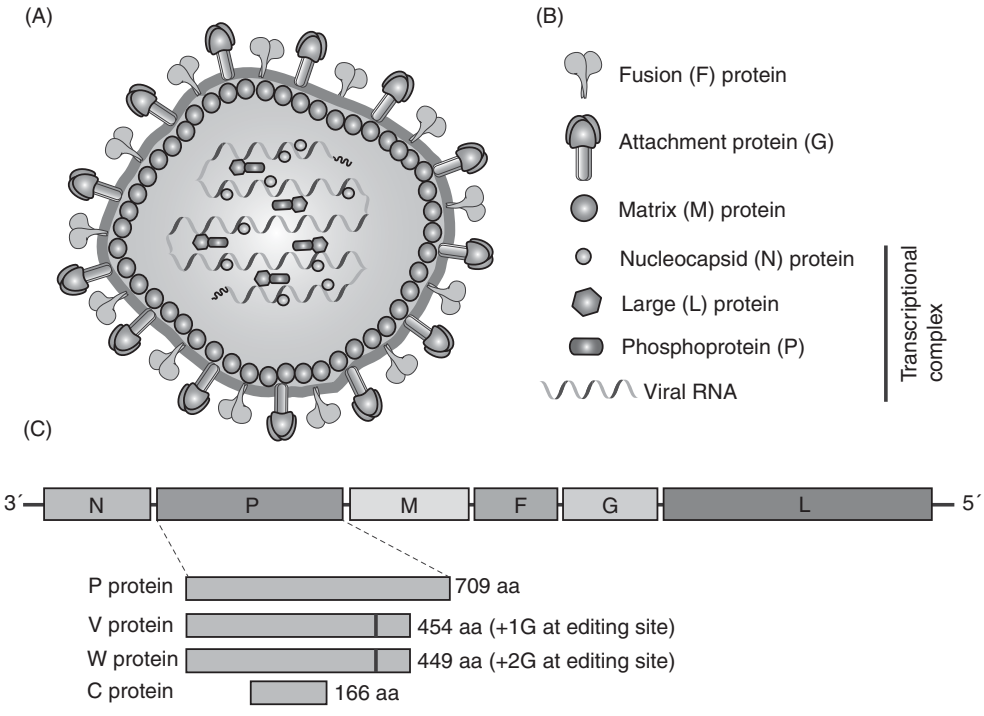

Fig. 3.1. Structure of Henipaviruses and their genome organization. (A, B) Schematic structure of Henipaviruses and arrangement of the viral proteins (C) The negative-sense genomic RNA of Henipaviruses is presented here in the 3′ to 5′ orientation. All genes, except the P gene, are monocistronic. The P gene encodes for three non-structural proteins: V, W and C, which are 454 aa, 449 and 166 aa long, respectively.

wingspan of up to 1.7 m, live in colonies of thousands of individuals and feed mainly on fruits and flowers, which they locate by smell. Once fruit bats were identified as the main reservoir for Henipaviruses, serological studies and immunosurveillance showed the circulation of Henipaviruses among the fruit bat population of the whole South-east Asia and Oceania. Recent studies revealed the circulation of Henipavirus-like viruses in Africa, where clinical cases have not yet being registered. Deforestation and agriculture intensification led to a decrease of bats' natural habitat and represent critical factors responsible for the crossing of the species barrier (Pulliam *et al.*, 2012). In this context, farm and domestic animals such as pigs or horses were more easily exposed to bat droppings, which are the primary source of contamination. Evidence of NiV infection was also found in cats, dogs, horses, cows and goats, but was mainly restricted to dogs for HeV (Geisbert *et al.*, 2012). The respiratory form of the disease in domestic animals, especially in horses and pigs, induces high production of contaminating aerosol particles and allows transmission to humans. Concerning NiV, two other ways of human contamination were discovered in Bangladesh. The first suggests the drinking of raw palm sap contaminated with bat saliva and/or droppings (Salah Uddin Khan *et al.*, 2011) and the second concerns human-to-human transmission through aerosol in the case of close contact with infected patients (Homaira *et al.*, 2010).

3.2 Pathogenesis

3.2.1 Clinical signs

In humans, NiV and HeV show similar clinical symptoms, although much more is known about the pathogenesis of NiV, given

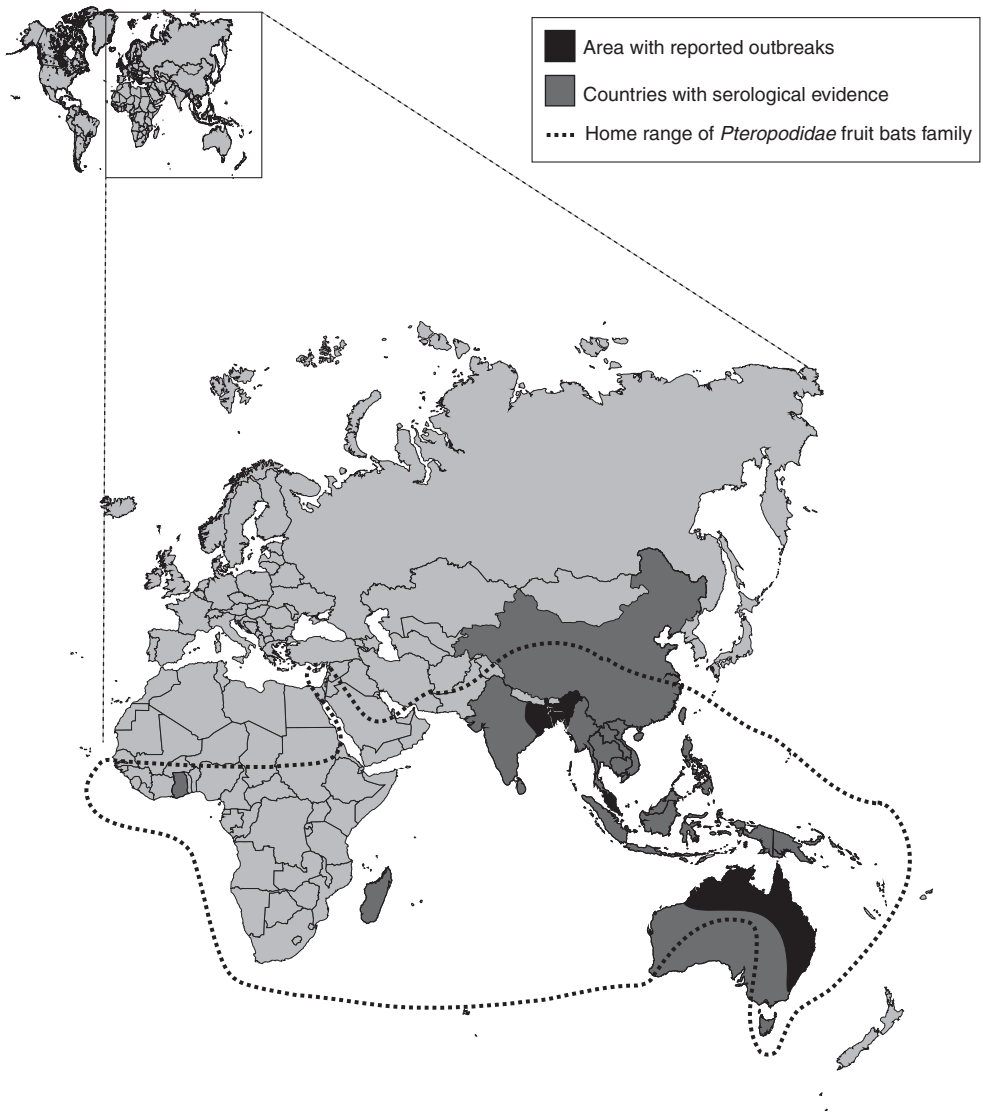

Fig. 3.2. Flying foxes, their distribution and the locations of disease outbreaks caused by Hendra virus and Nipah virus. The geographic distribution of the viruses strongly correlates with the animals' home range.

the larger number of human cases with this virus. After an incubation period of up to 2 weeks, patients develop flu-like symptoms, frequently followed by fatal encephalitis with multiple organ failure. The neurological symptoms may be associated with a pulmonary syndrome. NiV lethality ranges from 40 to 100% and HeV lethality varies with a similar range. Relapse and late-onset disease, even years after first exposure, was documented for both viruses (Wong and Tan, 2012).

In naturally infected animals, and especially in pigs and horses, pulmonary symptoms are more prominent. In pigs infected by NiV, the morbidity rate rises to 100% and the mortality rate varies from 1 to 40%, depending on the age. In many cases, the symptoms appear to be extremely subtle, but in animals that clearly develop clinical

signs, a combination of neurological and respiratory syndrome was observed. In porkers, the respiratory syndrome leads to a harsh non-productive cough, a condition commonly termed 'barking pig syndrome' and is considered to be the first characteristic of the disease. In sows, infection is usually related to sudden death or febrile syndrome (>39.9°C), followed by agitation and head pressing, champing of the mouth, drooling, nystagmus, myoclonus, tetanus-like spasm and seizures, varying degrees of rear leg paresis and pharyngeal muscle paralysis. In piglets, it remains unclear whether the high mortality rate is due to the viral infection itself or the inability of sows to nurse their litter (Mohd Nor *et al.*, 2000). In HeV infected horses, the clinical course of the disease usually lasts less than 36 hours after an incubation period up to 12 days. The disease starts with an acute febrile syndrome (>40°C) followed by an increase in the respiratory and heart rates. The disease progresses to neurological (head pressing, head tilt, ataxia and circling) and respiratory signs (laboured breathing and frothy nasal discharge) (Field *et al.*, 2010). In other domestic species such as dogs and cats, HeV infections are usually asymptomatic, but some infected animals may present similar respiratory and neurological clinical signs as in equines (Mills *et al.*, 2009).

3.2.2 Pathobiology

The life cycle of Henipaviruses is quite similar to other viruses of the *Paramyxoviridae* family. However, they possess specific traits responsible for their high pathogenicity and their potent zoonotic properties. The broad tropism among mammalian species is related to their highly conserved cellular surface receptors, Ephrin B2 and B3, which are broadly distributed in many cell types including neurons and endothelial cells (Maisner *et al.*, 2009). In natural cases, the infection happens mainly via the oronasal route. In this case, the first replication site is located in the epithelium of the oronasal and upper respiratory tract. Then, the virus uses leukocytes as conveyors to reach the second replication site, endothelial cells of the blood vessels, which strengthens the systemic dissemination (Mathieu *et al.*, 2011). Thus, vasculitis is one of the major features observed in tissues as well as thrombosis and parenchymal necrosis. Studies in pigs suggest that Henipaviruses could use two different routes to invade the central nervous system, involving either a breach of the blood–brain barrier or neuroinvasion via cranial nerves. Once it reaches the brain, the virus can spread from neuron to neuron and cause focal destruction of the parenchyma as observed in the MRIs of infected patients. Indeed, Henipaviruses are cytopathogenic viruses that cause cell-to-cell fusion leading to the formation of giant multinucleated cells called syncytia. These syncytia are responsible for the death of cells and the subsequent vascular breach, haemorrhages and necrosis observed in tissues. The inflammation that follows, induced by necrosis and thrombotic areas, leads to the recruitment of leukocytes in the brain that participate in the development of the meningo-encephalitis observed in histology (Wong and Tan, 2012).

The first immune mechanism to be triggered after pathogen entry is the type-I interferon (IFN-I) pathway. After its entry into the host cell, the viral genome is sensed by cytoplasmic RIG-like-receptors (RLR) – such as RIG-I or MDA5 – or by membranous toll-like-receptors (TLR) such as TLR3. This recognition leads to the phosphorylation and activation of several transcription factors, such as interferon regulatory factor 3 (IRF3), that translocate into the nucleus to stimulate the transcription of IFN-I genes. IFN-I is synthesized and released in the extracellular compartment (Fig. 3.3A). The released IFN activates surrounding cells, either by autocrine or paracrine means, expressing the IFN-I receptor (IFNAR). On its activation, IFNAR will phosphorylate signaling molecules, STAT1 and STAT2, leading to their dimerization. The STAT1/STAT2 dimer is translocated to the nucleus, where it activates the transcription of more than 300 IFN-stimulated genes (ISG) responsible for the production of proteins with antiviral properties such as PKR, OAS or Mx (Fig. 3.3B). In addition to their severe

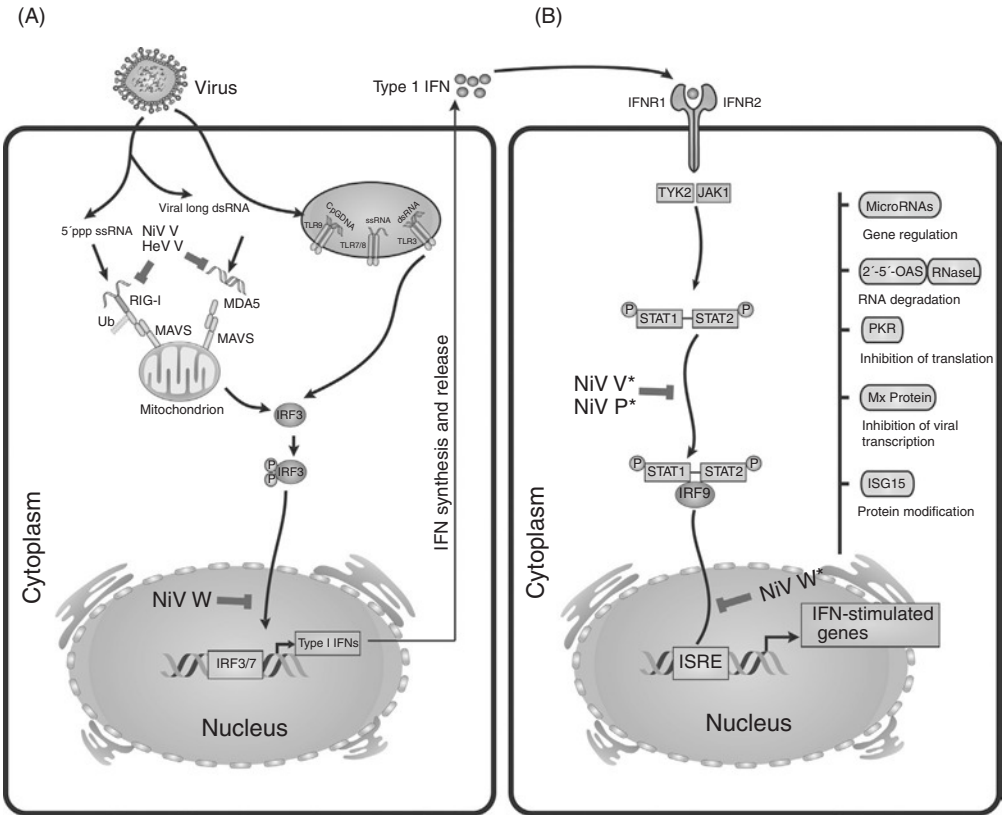

Fig. 3.3. IFN induction pathway and its inhibition by Henipaviruses. (A) Non-structural proteins V and W were shown to interfere with IFN induction at different levels. When overexpressed V, W and P protein could also inhibit IFN signaling (B), although this issue is still not completely resolved and the exact mechanism of complex interactions between Henipavirus and innate immune system remains to be understood. *Effects of viral proteins when overexpressed in cells remains to be confirmed during natural infection.

cytopathic effects, Henipaviruses are able to counteract this fine-tuned mechanism and have evolved strategies to control the IFN pathway. Indeed, the four proteins encoded by the P gene (P, V, W and C) could inhibit IFN-I signaling by different strategies. The V and W proteins have the ability to block the signaling pathway that leads to the activation of IFN-I production in the cytoplasm and in the nucleus, respectively. It seems that, regarding its nuclear localization, the W protein is much more efficient at blocking (Shaw *et al.*, 2005) than the V protein. The V protein was also demonstrated to inhibit the activity of melanoma differentiation-associated protein 5 (Mda5) and laboratory of genetics and physiology 2 (LGP2) helicases, two sensors of dsRNA involved in triggering IFN-I production (Parisien *et al.*, 2009). The precise action of the C protein still remains unknown, although it has been recently shown that it could regulate the production of proinflammatory cytokines in an infected host (Mathieu *et al.*, 2012). When overexpressed in cells, these NiV proteins (P, V and W proteins) are also able to block IFN-I signaling by disturbing the formation and trafficking of STAT1/STAT2 dimers (Basler, 2012). Because STAT plays an important role in the activation of ISG, responsible for the production of antiviral proteins, inactivation of STATs has important consequences on the immune response. However, when

observed in infected cells, the inhibition of IFN-I signaling pathways is not as strong as in overexpression experiments (Virtue *et al.*, 2011). This suggests that in natural conditions the levels of P, V, W and C proteins are not sufficient enough to block IFN-I signaling. Therefore, the precise mechanism used by Henipavirus proteins to counteract the innate immune system remains to be understood.

3.3 Diagnosis

Early diagnosis is a key issue to control disease expansion. Besides the clinical diagnosis, laboratory confirmation is essential, considering the huge health and economic implications that a confirmed case could represent. In 1999, in Malaysia, 1.1 million pigs were slaughtered and 956 farms were destroyed, representing a loss of 126.8 million euros for the Malaysian economy (Hosono *et al.*, 2006) and causing the unemployment of 31,800 people (Lam, 2003). Laboratory diagnoses are ruled by regularly updated guidelines, edited by the World Organization for Animal Health (OIE), in the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (Anonymous, 2008).

3.3.1 Sampling and submission of samples

The accurate detection of NiV and HeV must be supported by an appropriate sampling procedure, which requires a comprehensive understanding of viral pathobiology. Indeed, the collection site will depend on the time after exposure: sample collection from excretion or tissues for agent identification may be possible during clinical exam or necropsy, whereas blood collection for serological analysis will be relevant 10–14 days after infection. Authorities recommend systematic samples from brain, lungs, kidney and spleen. Samples from mediastinal lymph nodes and, in pregnant animals, uterus, placenta and foetal tissues may also be of great diagnosis value. Samples should be transported at 4°C if they can arrive at the reference laboratory within 48 hours or be frozen using dry ice or liquid nitrogen if the shipping period is longer than 48 hours. However, samples should not be kept at −20°C for a long period.

Because Henipaviruses are classified as BSL-4 microorganisms, their transportation must comply with the International Air Transport Association Dangerous Goods Regulations and samples must be submitted to reference laboratories in specially designed containers.

3.3.2 Molecular diagnosis

Molecular tests give rapid results with very high sensitivity. They can be used for early detection in suspected animal outbreaks as well as for confirmation of human cases. Conventional polymerase chain reaction (PCR) was used with success for many years but was recently superseded by quantitative real-time PCR (qPCR), which is much more sensitive and specific. Several tests and primers have been developed, depending on the technology platform available, and the possibility of discriminating NiV from HeV. These primers are mostly based on the N gene, but also M, P and L genes (Feldman *et al.*, 2009).

Progress in next-generation sequencing (NGS) technologies allows massive sequencing of samples to detect emergence of new strains and control the genetic diversity among host isolates. Such analysis demonstrated that HeV outbreaks do not correlate with a particular isolate but seem to mainly depend on host and environmental factors (Smith *et al.*, 2011).

3.3.3 Virus identification

Virus isolation remains highly advisable to confirm a new outbreak or to identify a new host. However, given the high pathogenicity of both viruses, these experiments have to be carried out in BSL-4 laboratories. To obtain rapid results when such facilities are not available, experiments to

confirm suspicion may be conducted in a BSL-3 laboratory, but highly stringent local guidelines must be applied to protect operators and the population. If cytopathic effects (CPE) compatible with paramyxovirus-like infection are observed, all samples, cultured cells and supernatant have to be transferred to a BSL-4 laboratory. Henipaviruses grow very well to high titre on Vero cells. CPE usually develop after 3 days of culture, even if authorities recommend two passages, 5-days each, to declare the sample negative (Anonymous, 2008). At low multiplicity of infection, CPE are characterized by formation of giant multinucleated cells called syncytia that may contain over 60 nuclei aggregated in the middle or distributed in a ring along the membrane, depending on its maturity (Hyatt *et al.*, 2001). Once the virus has been identified, complementary methods such as qPCR or sequencing should be used for final confirmation and characterization.

Electron microscopy was used in the discovery of HeV (Murray *et al.*, 1995) and remains a helpful tool in diagnosis (Daniels *et al.*, 2001). Direct visualization of viruses is possible in supernatant of infected cells by negative contrast electron microscopy and detection of virus–antibody interaction is feasible by immunoelectron microscopy. This technique allows ultrastructural information to be collected that may help to complete the diagnosis. Ultrastructural studies can also be completed by the grid cell culture method (Hanna *et al.*, 2006) or identification of replicating viruses in thin sections of fixed, embedded cell cultures and infected tissues (Hyatt *et al.*, 2001).

Immunohistochemistry is certainly one of the most useful tests for Henipaviruses because it allows specific staining of the virus in formalin-fixed paraffin-embedded tissues. For that reason, it is recommended to sample organs taken at different anatomical levels, especially brain and lungs. The initial immunohistochemical investigations of HeV-infected tissue used a convalescent human serum, but a range of polyclonal and monoclonal antisera are now available. A study of staining efficiency of these different antisera showed that the hyperimmune rabbit sera raised against recombinant N proteins of NiV and HeV were the most effective (Wang and Daniels, 2012). This technique also allows retrospective diagnosis from archival material (Hooper *et al.*, 1996).

3.3.4 Serology

Considering the rapidity and the highly fatal outcome of Henipavirus infection, serological tests are not considered to be a very useful diagnostic tool for identifying cases during early phases of an outbreak. However, such tests may be of particular importance for immunosurveillance and assessment of Henipavirus circulation in wild and domestic animals. Serological tests helped to identify new potential hosts, such as dogs in Henipavirus-infected farms (Mills *et al.*, 2009), and to monitor Henipavirus distribution among its natural reservoir, fruit bats. These analyses also enabled the identification of 'Henipavirus-like' viruses circulating in African fruit bats (Drexler *et al.*, 2009), when neither human nor domestic animal clinical cases have been reported in this region to date.

To confirm the presence of Henipavirus-specific neutralizing antibodies following an exposure to the virus, the virus neutralization test (VNT) is still considered by authorities as the reference standard (Anonymous, 2008). This test needs to be performed in BSL-4 laboratories because it uses live viruses. Serial dilutions of tested sera are incubated in a 96-well plate with 50 plaque forming units of either NiV or HeV at 37°C. After a 1-hour incubation period, Vero cells are added to the wells. Three days later, cultures are read for detection of cytopathic effects (CPE). Sera that completely block development of CPE are declared positive for Henipavirus exposure. However, for rapid screening, another test based on immunostaining was developed to reduce the incubation period to 24 hours (Crameri *et al.*, 2002).

Because it does not require BSL-4 facilities, the Enzyme-Linked ImmunoSorbent Assay (ELISA) remains one of the most common tools for immunosurveillance and

epidemiological studies. Antigen is generally produced from non-ionic detergent treatment of infected Vero cells. To control for high levels of nonspecific binding activity, a control antigen from uninfected Vero cells must be used in comparison. The ratio between specific and non-specific binding activity gives the result. Various ELISA tests based on recombinant proteins, including NiV N (Chen *et al.*, 2006), NiV G (Eshaghi *et al.*, 2005) and its soluble form (Wang and Daniels, 2012), truncated forms of NiV and HeV P proteins (Chen *et al.*, 2007), were developed, but their usefulness in field diagnosis remains to be determined. ELISA tests developed by the Centers for Disease Control (CDC, Atlanta, USA), following the Malaysian outbreaks of NiV, allow not only for detection of IgG by indirect ELISA but also IgM by capture ELISA. However, such tests have a major drawback due to their suboptimal specificity. Indeed, the specificity of the indirect NiV ELISA was shown to be 98.4% (Ong *et al.*, 2000). During a NiV outbreak situation, this specificity is satisfactory enough because a high proportion of pigs are infected and the goal of the test is to identify infected farms. However, in the absence of an outbreak, a positive test should lead to immediate and drastic countermeasures to control the spread of the virus. In this case, the number of false positives would be unacceptable and result in dramatic waste. For that reason, all ELISA positive samples have to be confirmed by VNT in accredited BSL-4 laboratories.

The use of pseudotyped viruses represents a good alternative in order to avoid BSL-4 laboratory testing. They are genetically engineered viruses belonging to BSL-1 or BSL-2 groups that express either HeV or NiV envelope glycoproteins. They allow VNT to be performed in a BSL-2 laboratory, which increases the number of laboratories that may get an access to such tests. Currently, several pseudotyped systems have been developed for NiV. The first system, published in 2009, uses a recombinant VSV expressing NiV F and G on viral envelope and luciferase as the reporter molecule (Tamin *et al.*, 2009). The specificity appeared to be relatively good (94–100%) but sensitivity was not convincing enough for diagnosis application. The same year, a second VSV pseudotyped with NiV F and G, which expresses the green fluorescent protein (GFP) as a reporter molecule, was proposed (Kaku *et al.*, 2009). In this system, quantification was performed by either fluorescence measurement or GFP-expressing cell count. This system demonstrates sensitivity at least equal to conventional VNT. A third pseudotyped virus, using HIV lentivirus as vector and luciferase and GFP as the reporter molecules, was developed in 2010 (Khetawat and Broder, 2010). Although it provides information on virus–host interaction, its suitability to detect neutralizing antibodies in diagnosis was not assessed. In addition, another system based on murine leukemia virus bearing both NiV G and NiV F proteins with GFP as reporter gene was developed in Lyon, France (Mathieu *et al.*, 2011), and is currently being tested as a potential diagnostic tool. The last reported system was published in 2012 and is based on a second-generation pseudotyped VSV expressing the secreted alkaline phosphatase (SEAP) as the reporter molecule (Kaku *et al.*, 2012). The major improvement of this reporter is that neutralization titre can be easily obtained from measurement of SEAP activity in supernatant using a common ELISA plate reader. This system appears to be as precise as conventional VNT but only needs 2 µl of serum compared with the 50 µl used in conventional VNT. Thus, it allows significant quantities of valuable serum to be spared, increased throughput for diagnosis and experiments to be performed in a BSL-2 laboratory. This method with high sensitivity and specificity is not yet validated for definitive diagnosis but could be valuable on the front line.

The Luminex-based binding test was developed in 2007 with the major advantage that it does not require live cell culture and can be done in a standard laboratory, without any biocontainment (Bossart *et al.*, 2007). Soluble HeV and NiV G (sG) proteins were coated on two beads with distinctive fluorescent spectra. A predetermined number of each type of beads was mixed with serial dilutions of test sera. After an incubation

period of 30 minutes, bound antibodies were revaluated by addition of biotinylated A and G proteins followed by streptavidin/phycoerythrin. The final fluorescence measurement was performed using a Bio-Plex Protein Array System (BioRad, USA). Pseudo-neutralization assays are possible by replacing biotinylated A and G proteins with a biotinylated soluble ephrin-B2 molecule, known as the natural partner of HeV-G and NiV-G proteins. A very high sensitivity and the good specificity of this technique, associated with its innocuousness, places the Luminex assay as one of the best ways to conduct mass Henipavirus serosurveillance. However, this test does not replace the established reference protocol in serological tests for Henipaviruses: ELISA tests confirmed by VNT (Daniels *et al.*, 2001).

3.4 Prophylactic and Therapeutic Perspectives

3.4.1 Prophylaxis

Prophylaxis is currently the major way to control Henipavirus infections even if medical prophylaxis is still at an experimental level. Because exposure to infectious bat droppings is the major source of contamination, the most efficient prophylaxis method is still environmental. It is very difficult to control deforestation in these regions; however, avoiding the overlap between orchards and pig-pens in Malaysia was a good measure to prevent the re-emergence of the disease. In Bangladesh, where the major contamination source is consumption of palm sap, the use of a bamboo skirt to cover the collection site and make it inaccessible to bats significantly reduced the risk of contamination (Nahar *et al.*, 2010). However, covering each tree is a time-consuming process and this method, although it is currently the best existing prophylaxis in Bangladesh, is not used regularly.

Sanitary prophylaxis is also very efficient but usually implies that an outbreak has already been identified. It consists of a massive awareness-raising campaign in the concerned population about good hygiene practice. For example, during the Malaysian outbreak, the recommendations were broadcast through mass media such as television, radio, schools and newspapers. Malaysian authorities also published posters, leaflets and booklets in various languages to inform the population at risk. They also created a website and a hotline dedicated to providing information to the general public (Chua, 2010). Because Henipaviruses are enveloped viruses, they are very sensitive to detergent. Intensive use of soap and detergent is a cost-effective measure to reduce possible infection in people through hand washing and washing tools (knives, wheelbarrow, vehicles and medical instruments) in contact with infectious materials. For people who could be accidentally exposed to infectious particles, disposable long-sleeved overalls, gloves, masks, protective glasses and boots is highly recommended.

Medical prophylaxis against Henipaviruses mostly consists of vaccines. Some vaccines are under development for animals. The research on live recombinant vaccines gave first results in 2004 with a recombinant vaccinia virus expressing either NiV F or G proteins (Guillaume *et al.*, 2004). However, even if the protection of golden hamsters was clearly demonstrated, the use of such vaccine vector in humans is unlikely. For livestock, a vaccine candidate using recombinant canarypox virus (ALVAC) expressing either NiV F or G glycoproteins was tested on pigs in 2006. This vaccine elicited a good neutralizing antibody titre and prevented disease and virus shedding in vaccinated animals (Weingart *et al.*, 2006). Research is ongoing for a similar vaccine against HeV in horses. In comparison with live attenuated vaccines, subunit immunogen-based vaccines are a more suitable option for vaccine development, especially in humans. They are simple to make, safe concerning infection risk and relatively quick to implement. A vaccine using a soluble form of NiV or HeV G protein, combined with the appropriate adjuvant (Montanide, Quil A and DEAE-Dextran), showed very good results in cats (Mungall *et al.*, 2006), ferrets (Pallister *et al.*, 2011)

and more recently in African green monkeys (Broder *et al.*, 2012). The HeV-sG subunit was licensed by a multinational animal health company and should be commercially available in 2013 for horses. The next challenge in vaccine development is to implement a method that allows serological differentiation of infected from vaccinated animals.

3.4.2 Treatment

Considering the current small annual number of human cases, development of efficient treatments seems to be a more suitable option than production of a human vaccine. In the initial Malaysian outbreak of 1998, ribavirin, a ribonucleoside analog with broad antiviral activity, was used on 140 patients (Chong *et al.*, 2001). Compared with the patients who were admitted before availability of this drug or who refused the treatment, mortality in ribavirin-treated patients was 36% less. However, laboratory experiments on animal models, such as golden hamster, did not confirm the efficiency of ribavirin in the treatment of Henipavirus infection (Georges-Courbot *et al.*, 2006; Freiberg *et al.*, 2010) and ribavirin did not prove effective in the treatment of HeV-infected patients (Playford *et al.*, 2010). Until now, interferon, a commercially available antiviral drug with broad activity, was not considered a good candidate because NiV and HeV were known to inhibit the interferon pathway. However, recently it has been shown that, in the context of human cell infection, Henipaviruses are able to block interferon production but not signaling (Virtue *et al.*, 2011). Such results indicate a therapeutic potential of interferon as a post-exposure treatment.

To compensate for the lack of appropriate drugs, many strategies have been developed. One of them consists of using peptides derived from C-terminal heptad repeat regions of a paramyxovirus F glycoprotein. These peptides are able to inhibit the fusion process between the viral envelope and the membrane of the target cell. Concerning Henipaviruses, the HR-peptides that proved most efficient are derived from another paramyxovirus, the human parainfluenza virus type 3 (hPIV3). The *in vivo* efficiency of this peptide was enhanced by sequence optimization and adjunction of a cholesterol tag that increased the capacity of the peptide to reach the central nervous system where the virus produces most damage (Porotto *et al.*, 2010). Such peptides represent a good therapeutic approach for treating people before encephalitic manifestations.

An original attempt to decrease viral load was done with protocells. These are small nanoporous silica beads enveloped by a lipid bilayer and bearing a Henipavirus receptor, ephrinB2 (Porotto *et al.*, 2011). Circulating virus is attracted to the protocells decoy and the fusion mechanism is triggered, which prevents the virus from infecting host cells. Despite the ingenuity of this system, the use of protocells in infected patients may not be as relevant as expected because of the low amount of free-circulating virus.

Passive immunization is currently the most achieved therapeutic strategy. The proof-of-concept that immunotherapy could prevent Henipavirus infection was rapidly established in the hamster model. The first evidence of passive protection against NiV was shown with monospecific NiV G and NiV F polyclonal antibodies produced in hamsters using recombinant vaccinia viruses. Two intraperitoneal administrations of this serum, 1 hour before challenge and 24 hours after infection, allowed the survival of 100% of treated hamsters (Guillaume *et al.*, 2004). Murine monoclonal antibodies (mAbs) against NiV F and NiV G were next developed as ascitic fluid preparations and tried in the hamster model (Guillaume *et al.*, 2006). When used in the same conditions as hamster polyclonal antibodies, murine mAbs showed similar results and protected 100% of treated animals. When the mAbs treatment was done 24 hours after challenge, the protection level decreased to 50%. Similar studies were done with HeV challenge and proved to be efficient when administered before challenge, thus demonstrating the good cross-reaction of murine mAbs targeted

against NiV F protein (Guillaume *et al.*, 2009). Altogether, these studies proved that prophylactic passive immunotherapy directed against Henipavirus glycoproteins is possible. However, such antibodies would answer much better the real field need if they could be given after Henipavirus exposure. The development of such antibodies, suitable for humans, was allowed by recombinant antibody methods. This technology uses a large naïve human phage display combinatorial antibody library that allows isolation of Fab fragments of single chain variable fragments. Neutralizing human mAbs specific against the Henipavirus G glycoproteins were identified (Zhu *et al.*, 2006) and two of them were converted to full-length human IgG1 antibodies. One of these recombinant antibodies, m102.4, possesses excellent inhibitory activity against NiV but also a potent cross-reactivity towards HeV with 50% inhibitory concentrations below 0.04 μg/ml for NiV and 0.6 μg/ml for HeV (Zhu *et al.*, 2008). Such encouraging results led to application tests in animal models. First, the human mAb 102.4 was assessed in the ferret model either 24 hours before or 10 hours after NiV challenge (Bossart *et al.*, 2009). All animals from the post-treated group survived, whereas 2 of 3 animals from the pre-treated group died, suggesting the rapid clearance of mAb 102.4 antibodies in ferrets. Nevertheless, this study is the first to report an effective post-exposure treatment for Henipavirus infection. In accordance with requirements for use of drugs in humans, m102.4 antibody was tested in a second animal species, the African green monkey. Animals were challenged with HeV and treated with a 20 mg/kg dose of m102.4 beginning at 10 hours, 24 hours or 72 hours post infection. They received a second identical dose 48 hours after the first treatment. All the treated animals survived the challenge, even if animals of the 72-hour treatment group experienced some transient neurological problems. There was no evidence of HeV-specific pathology in organs of any treated animal at the end of the protocol (40 days post infection). Although very high dose of mAb was required for the injection, these studies reveal its high potential as a post-exposure treatment for Henipaviruses.

3.5 Conclusions

Infectious diseases are a key concern in public opinion. Emergence of new viruses such as H5N1 influenza virus or SARS coronavirus raises the threat of deadly pandemics. Among these emergent viruses, Henipaviruses attract particular attention among members of the *Paramyxovirus* family, because they possess a high zoonotic potential associated with one of the highest fatality rates observed in infectious diseases. Economic consequences of an outbreak are very serious and the estimated loss subsequent to the initial Malaysian outbreak was estimated to be over US$170 million. Because there is currently no available efficient prophylaxis or treatment, study of these viruses is restricted to the few BSL-4 laboratories existing in the world. Such restrictions slow down scientific breakthroughs, but many trails have been blazed since the first emergence of HeV in 1996. Early diagnosis associating clinical and laboratory assessment is the key issue for their control. Considering that 70% of emerging or re-emerging infectious diseases possess a zoonotic or vector-borne origin, the necessity to gather competencies around the 'One World One Health Initiative' is a critical factor to enhance public health efficacy. In this context, field veterinarians, physicians as well as researchers represent the keystones of the surveillance programme and will allow synergistic and rapid expansion in scientific knowledge. Within this context, Henipaviruses will represent a significant challenge for the coming years and research on these viruses should improve public safety towards potential pandemics or bioterrorist attack.

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References

Anonymous (2008) *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals: Mammals, Birds, and Bees*, 6th edn. OIE, Paris.

Basler, C.F. (2012) Nipah and Hendra virus interactions with the innate immune system. *Current Topics in Microbiology and Immunology* 359, 123–152.

Bossart, K.N., McEachern, J.A., Hickey, A.C., Choudhry, V., Dimitrov, D.S., Eaton, B.T. and Wang, Lin-Fa (2007) Neutralization assays for differential henipavirus serology using Bio-Plex protein array systems. *Journal of Virological Methods* 142, 29–40.

Bossart, K.N., Zhu, Z., Middleton, D., Klippel, J., Crameri, G., Bingham, J., McEachern, J.A., Green, D., Hancock, T.J., Chan, Y.-P., Hickey, A.C., Dimitrov, D.S., Wang, Lin-Fa and Broder, C.C. (2009) A neutralizing human monoclonal antibody protects against lethal disease in a new ferret model of acute Nipah virus infection. *PLoS Pathogens* 5(10) DOI: 10.1371/journal.ppat.1000642.

Broder, C.C., Geisbert, T.W., Xu, K., Nikolov, D.B., Wang, Lin-Fa, Middleton, D., Pallister, J. and Bossart, K.N. (2012) Immunization strategies against henipaviruses. *Current Topics in Microbiology and Immunology* 359, 197–223.

Chen, J.-M., Yu, M., Morrissy, C., Zhao, Y.-G., Meehan, G., Sun, Y.-X., Wang, Q.-H., Zhang, W., Wang, Lin-Fa and Wang, Z.-L. (2006) A comparative indirect ELISA for the detection of henipavirus antibodies based on a recombinant nucleocapsid protein expressed in *Escherichia coli. Journal of Virological Methods* 136, 273–276.

Chen, J.-M., Yaiw, K.C., Yu, M., Wang, Lin-Fa, Wang, Q.-H., Crameri, G. and Wang, Z.-L. (2007) Expression of truncated phosphoproteins of Nipah virus and Hendra virus in *Escherichia coli* for the differentiation of henipavirus infections. *Biotechnology Letters* 29, 871–875.

Chong, H.T., Kamarulzaman, A., Tan, C.T., Goh, K.J., Thayaparan, T., Kunjapan, S.R., Chew, N.K., Chua, K.B. and Lam, S.K. (2001) Treatment of acute Nipah encephalitis with ribavirin. *Annals of Neurology* 49, 810–813.

Chua, K.B. (2010) Risk factors, prevention and communication strategy during Nipah virus outbreak in Malaysia. *Malaysian Journal of Pathology* 32, 75–80.

Crameri, G., Wang, L.F., Morrissy, C., White, J. and Eaton, B.T. (2002) A rapid immune plaque assay for the detection of Hendra and Nipah viruses and anti-virus antibodies. *Journal of Virological Methods* 99, 41–51.

Daniels, P., Ksiazek, T. and Eaton, B.T. (2001) Laboratory diagnosis of Nipah and Hendra virus infections. *Microbes and Infection/Institut Pasteur* 3, 289–295.

Drexler, J.F., Corman, V.M., Gloza-Rausch, F., Seebens, A., Annan, A., Ipsen, A., Kruppa, T., Müller, M.A., Kalko, E.K.V., Adu-Sarkodie, Y., Oppong, S. and Drosten, C. (2009) Henipavirus RNA in African bats. *PLoS One* 4, e6367.

Eaton, B.T., Broder, C.C., Middleton, D. and Wang, Lin-Fa (2006) Hendra and Nipah viruses: different and dangerous. *Nature Reviews Microbiology* 4, 23–35.

Eshaghi, M., Tan, W.S., Chin, W.K. and Yusoff, K. (2005) Purification of the extra-cellular domain of Nipah virus glycoprotein produced in *Escherichia coli* and possible application in diagnosis. *Journal of Biotechnology* 116, 221–226.

Feldman, K.S., Foord, A., Heine, H.G., Smith, I.L., Boyd, V., Marsh, G.A., Wood, J.L.N., Cunningham, A.A. and Wang, L.-F. (2009) Design and evaluation of consensus PCR assays for henipaviruses. *Journal of Virological Methods* 161, 52–57.

Field, H., Schaaf, K., Kung, N., Simon, C., Waltisbuhl, D., Hobert, H., Moore, Frederick, Middleton, D., Crook, A., Smith, G., Daniels, P., Glanville, R. and Lovell, D. (2010) Hendra virus outbreak with novel clinical features, Australia. *Emerging Infectious Diseases* 16, 338–340.

Freiberg, A.N., Worthy, M.N., Lee, B. and Holbrook, M.R. (2010) Combined chloroquine and ribavirin treatment does not prevent death in a hamster model of Nipah and Hendra virus infection. *Journal of General Virology* 91, 765–772.

Geisbert, T.W., Feldmann, H. and Broder, C.C. (2012) Animal challenge models of henipavirus infection and pathogenesis. *Current Topics in Microbiology and Immunology* DOI: 10.1007/82_2012_208.

Georges-Courbot, M.C., Contamin, H., Faure, C., Loth, P., Baize, S., Leyssen, P., Neyts, J. and Deubel, V. (2006) Poly(I)-poly(C12U) but not ribavirin prevents death in a hamster model of Nipah virus infection. *Antimicrobial Agents and Chemotherapy* 50, 1768–1772.

Guillaume, V., Contamin, H., Loth, P., Georges-Courbot, M.C., Lefeuvre, A., Marianneau, P., Chua, K.B., Lam, S.K., Buckland, R., Deubel, V. and Wild, T.F. (2004) Nipah virus: vaccination and passive protection studies in a hamster model. *Journal of Virology* 78, 834–840.

Guillaume, V., Contamin, H., Loth, P., Grosjean, I., Georges-Courbot, M.C., Deubel, V., Buckland, R. and Wild, T.F. (2006) Antibody prophylaxis and therapy against Nipah virus infection in hamsters. *Journal of Virology* 80, 1972–1978.

Guillaume, V., Wong, K.T., Looi, R.Y., Georges-Courbot, M.C., Barrot, L., Buckland, R., Wild, T.F. and Horvat, B. (2009) Acute Hendra virus infection: analysis of the pathogenesis and passive antibody protection in the hamster model. *Virology* 387, 459–465.

Hanna, J.N., McBride, W.J., Brookes, D.L., Shield, J., Taylor, C.T., Smith, I.L., Craig, S.B. and Smith, G.A. (2006) Hendra virus infection in a veterinarian. *The Medical Journal of Australia* 185, 562–564.

Homaira, N., Rahman, M., Hossain, M.J., Epstein, J.H., Sultana, R., Khan, M.S.U., Podder, G., Nahar, K., Ahmed, B., Gurley, E.S., Daszak, P., Lipkin, W.I., Rollin, P.E., Comer, J.A., Ksiazek, T.G. and Luby, S.P. (2010) Nipah virus outbreak with person-to-person transmission in a district of Bangladesh, 2007. *Epidemiology and Infection* 138, 1630–1636.

Hooper, P.T., Gould, A.R., Russell, G.M., Kattenbelt, J.A. and Mitchell, G. (1996) The retrospective diagnosis of a second outbreak of equine morbillivirus infection. *Australian Veterinary Journal* 74, 244–245.

Hosono, H., Kono, H., Ito, S. and Shirai, J. (2006) T6-1.3.1 – Economic impact of nipah virus infection outbreak in Malaysia. International Symposia on Veterinary Epidemiology and Economics (ISVEE) proceedings, ISVEE 11: *Proceedings of the 11th Symposium of the International Society for Veterinary Epidemiology and Economics*, Cairns, Australia (Theme 6 - Global response and emerging diseases: Wildlife disease session), p. 324.

Hyatt, A.D., Zaki, S.R., Goldsmith, C.S., Wise, T.G. and Hengstberger, S.G. (2001) Ultrastructure of Hendra virus and Nipah virus within cultured cells and host animals. *Microbes and Infection* 3, 297–306.

Kaku, Y., Noguchi, A., Marsh, G.A, McEachern, J.A., Okutani, A., Hotta, K., Bazartseren, B., Fukushi, S., Broder, C.C., Yamada, A., Inoue, S. and Wang, L.F. (2009) A neutralization test for specific detection of Nipah virus antibodies using pseudotyped vesicular stomatitis virus expressing green fluorescent protein. *Journal of Virological Methods* 160, 7–13.

Kaku, Y., Noguchi, A., Marsh, G.A., Barr, J.A., Okutani, A., Hotta, K., Bazartseren, B., Fukushi, S., Broder, C.C., Yamada, A., Inoue, S. and Wang, L.F. (2012) Second generation of pseudotype-based serum neutralization assay for Nipah virus antibodies: sensitive and high-throughput analysis utilizing secreted alkaline phosphatase. *Journal of Virological Methods* 179, 226–232.

Khetawat, D. and Broder, C.C. (2010) A functional henipavirus envelope glycoprotein pseudotyped lentivirus assay system. *Virology Journal* 7, 312.

Lam, S.-K. (2003) Nipah virus – a potential agent of bioterrorism? *Antiviral Research* 57, 113–119.

Maisner, A., Neufeld, J. and Weingartl, H. (2009) Organ- and endotheliotropism of Nipah virus infections in vivo and in vitro. *Thrombosis and Haemostasis* 102, 1014–1023.

Mathieu, C., Pohl, C., Szecsi, J., Trajkovic-Bodennec, S., Devergnas, S., Raoul, H., Cosset, F.-L., Gerlier, D., Wild, T.F. and Horvat, B. (2011) Nipah virus uses leukocytes for efficient dissemination within a host. *Journal of Virology* 18, 85, 7863–7871.

Mathieu, C., Guillaume, V., Volchkova, V.A., Pohl, C., Jacquot, F., Looi, R.Y., Wong, K.T., Legras-Lachuer, C. Volchkov, V.E., Lachuer, J. and Horvat, B. (2012) Nonstructural Nipah virus C protein regulates both the early host proinflammatory response and viral virulence. *Journal of Virology* in press.

Mills, J.N., Alim, A.N.M., Bunning, M.L., Lee, O.B., Wagoner, K.D., Amman, B.R., Stockton, P.C. and Ksiazek, T.G. (2009) Nipah virus infection in dogs, Malaysia, 1999. *Emerging Infectious Diseases* 15, 950–952.

Mohd Nor, M.N., Gan, C.H. and Ong, B.L. (2000) Nipah virus infection of pigs in peninsular Malaysia. *Revue Scientifique et Technique (International Office of Epizootics)* 19, 160–165.

Mungall, B.A., Middleton, D., Crameri, G., Bingham, J., Halpin, K., Russell, G., Green, D., McEachern, J., Pritchard, L.I., Eaton, Bryan T., Wang, L.F., Bossart, K.N. and Broder, C.C. (2006) Feline model of acute nipah virus infection and protection with a soluble glycoprotein-based subunit vaccine. *Journal of Virology* 80, 12,293–12,302.

Murray, K., Rogers, R., Selvey, L., Selleck, P., Hyatt, A., Gould, A., Gleeson, L., Hooper, P. and Westbury, H. (1995) A novel morbillivirus pneumonia of horses and its transmission to humans. *Emerging Infectious Diseases* 1, 31–33.

Nahar, N., Sultana, R., Gurley, E.S., Hossain, M.J. and Luby, S.P. (2010) Date palm sap collection: exploring opportunities to prevent Nipah transmission. *EcoHealth* 7, 196–203.

Ong, B.L., Daniels, P.W., Bunning, M., Jamaluddin, A., White, J., Muniandy, M., Olson, J. and Chang, K.W. (2000) A surveillance programme for the detection of pig herds exposed to nipah virus infections in Peninsula Malaysia. International Symposia on Veterinary Epidemiology and Economics (ISVEE) proceedings, ISVEE 9: *Proceedings of the 9th Symposium of the International Society*

for Veterinary Epidemiology and Economics, Breckenridge, Colorado, USA (Surveillance and monitoring session), p. 263.

Pallister, J., Middleton, D., Wang, Lin-Fa, Klein, R., Haining, J., Robinson, R., Yamada, M., White, J., Payne, J., Feng, Y.-R., Chan, Y.-P. and Broder, C.C. (2011) A recombinant Hendra virus G glycoprotein-based subunit vaccine protects ferrets from lethal Hendra virus challenge. *Vaccine* 29, 5623–5630.

Parisien, J.-P., Bamming, D., Komuro, A., Ramachandran, A., Rodriguez, J.J., Barber, G., Wojahn, R.D. and Horvath, C.M. (2009) A shared interface mediates paramyxovirus interference with antiviral RNA helicases MDA5 and LGP2. *Journal of Virology* 83, 7252–7260.

Playford, E.G., McCall, B., Smith, G., Slinko, V., Allen, G., Smith, I., Moore, F., Taylor, C., Kung, Y.H. and Field, H. (2010) Human Hendra virus encephalitis associated with equine outbreak, Australia, 2008. *Emerging Infectious Diseases* 16, 219–223.

Porotto, M., Rockx, B., Yokoyama, C.C., Talekar, A., DeVito, I., Palermo, L.M., Liu, J., Cortese, R., Lu, M., Feldmann, H., Pessi, A. and Moscona, A. (2010) Inhibition of Nipah virus infection in vivo: targeting an early stage of paramyxovirus fusion activation during viral entry. *PLoS Pathogens* 6, e1001168.

Porotto, M., Yi, F., Moscona, A. and LaVan, D.A. (2011) Synthetic protocells interact with viral nanomachinery and inactivate pathogenic human virus. *PLoS ONE* 6, e16874.

Pulliam, J.R.C., Epstein, J.H., Dushoff, J., Rahman, S.A., Bunning, M., Jamaluddin, A.A., Hyatt, A.D., Field, H.E., Dobson, A.P. and Daszak, P. (2012) Agricultural intensification, priming for persistence and the emergence of Nipah virus: a lethal bat-borne zoonosis. *Journal of the Royal Society, Interface/the Royal Society* 9, 89–101.

Salah Uddin Khan, M., Hossain, J., Gurley, E.S., Nahar, N., Sultana, R. and Luby, S.P. (2011) Use of infrared camera to understand bats' access to date palm sap: implications for preventing Nipah virus transmission. *EcoHealth* 7, 517–525.

Shaw, M.L., Cardenas, W.B., Zamarin, D., Palese, P. and Basler, C.F. (2005) Nuclear localization of the Nipah virus W protein allows for inhibition of both virus- and toll-like receptor 3-triggered signaling pathways. *Journal of Virology* 79, 6078–6088.

Smith, I., Broos, A., de Jong, C., Zeddeman, A., Smith, C., Smith, G., Moore, Fred, Barr, J., Crameri, G., Marsh, G., Tachedjian, M., Yu, M., Kung, Y.H., Wang, L.F. and Field, H. (2011) Identifying Hendra virus diversity in pteropid bats. *PLoS ONE* 6, e25275.

Tamin, A., Harcourt, B.H., Lo, M.K., Roth, J.A., Wolf, M.C., Lee, B., Weingartl, H., Audonnet, J.-C., Bellini, W.J. and Rota, P.A. (2009) Development of a neutralization assay for Nipah virus using pseudotype particles. *Journal of Virological Methods* 160, 1–6.

Virtue, E.R., Marsh, G.A. and Wang, L.F. (2011) Interferon signaling remains functional during henipavirus infection of human cell lines. *Journal of Virology* 85, 4031–4034.

Wang, L.F. and Daniels, P. (2012) Diagnosis of henipavirus infection: current capabilities and future directions. *Current Topics in Microbiology and Immunology* 359, 179–196.

Weingartl, H.M., Berhane, Y., Caswell, J.L., Loosmore, S., Audonnet, J.-C., Roth, J.A. and Czub, M. (2006) Recombinant Nipah virus vaccines protect pigs against challenge. *Journal of Virology* 80, 7929–7938.

Wong, K.T. and Tan, C.T. (2012) Clinical and pathological manifestations of human henipavirus infection. *Current Topics in Microbiology and Immunology* 359, 95–104.

Zhu, Z., Dimitrov, A.S., Bossart, K.N., Crameri, G., Bishop, K.A., Choudhry, V., Mungall, B.A., Feng, Y.-R., Choudhary, A., Zhang, M.-Y., Feng, Y., Wang, L.F., Xiao, X., Eaton, B.T., Broder, C.C. and Dimitrov, D.S. (2006) Potent neutralization of Hendra and Nipah viruses by human monoclonal antibodies. *Journal of Virology* 80, 891–899.

Zhu, Z., Bossart, K.N., Bishop, K.A., Crameri, G., Dimitrov, A.S., McEachern, J.A., Feng, Y., Middleton, D., Wang, L.F., Broder, C.C. and Dimitrov, D.S. (2008) Exceptionally potent cross-reactive neutralization of Nipah and Hendra viruses by a human monoclonal antibody. *Journal of Infectious Diseases* 197, 846–853.

4 Canine Distemper Virus

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4.1 Introduction

Canine distemper virus (CDV) infects a broad range of carnivores and causes a highly contagious acute disease with considerable mortality (Carré, 1905; Deem *et al.*, 2000). In addition to regular outbreaks in well-known host species such as dogs, foxes, raccoons and skunks, CDV has caused devastating outbreaks in the Tanzanian Serengeti National Park. The virus almost eradicated the endangered populations of Lake Baikal and Caspian seals (Visser *et al.*, 1990; Kennedy *et al.*, 2000), and was even isolated from collared peccaries and more recently different macaque species (Yoshikawa *et al.*, 1989; Appel *et al.*, 1991; Qiu *et al.*, 2011). The discovery of dolphin, porpoise and cetacean morbilliviruses, as well as phocine distemper virus, over the recent decades further underlines the importance of morbilliviruses as emerging veterinary pathogens (Hall, 1995; Saliki *et al.*, 2002).

4.2 CDV Genome and Strains

Morbilliviruses are enveloped negative-sense, single-stranded RNA viruses that belong to the *Paramyxoviridae* family in the order Mononegavirales (Pringle, 1997). In addition to CDV and measles virus (MeV), which only infects humans and certain non-human primates, the morbillivirus genus includes several pathogens of veterinary importance such as peste des petits ruminants virus and the recently eradicated rinderpest virus (Westover and Hughes, 2001; Robertshaw, 2010). The viral genome contains six genes that result in synthesis of eight proteins. In addition to the nucleo- (N), phospho- (P), matrix (M), fusion (F), haemagglutinin (H) and polymerase (L) proteins, which are part of the viral particle (Fig. 4.1), the P gene also encodes the accessory C and V proteins (Diallo, 1990; Lamb and Parks, 2007). The two surface glycoproteins F and H are part of the viral envelope and mediate viral entry into the host cell. Together with the viral RNA, the N, P and L proteins form the ribonucleoprotein complex (RNP), which represents the minimal replicative unit (Lamb and Parks, 2007). During assembly, the M protein leads to association of the RNP with the viral envelope, leading to budding of the viral particles from the plasma membrane (Iwasaki *et al.*, 2009).

Even though CDV strains fall into distinct phylogenetic clades based on their geographic origin and year of isolation, only one serotype has been found to date

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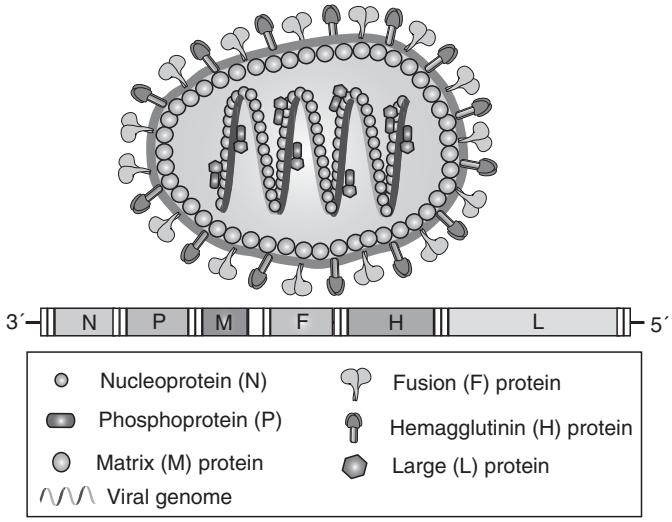

Fig. 4.1. Sketch of a canine distemper virus particle and genome organization.

(Bolt *et al.*, 1997; Zhao *et al.*, 2010; Gámiz *et al.*, 2011). The currently available live-attenuated vaccines are mostly based on wild-type isolates attenuated by serial passages in eggs or cell culture (Haig, 1956; Rockborn, 1959; Demeter *et al.*, 2010). These vaccines elicit protective immune responses against circulating wild-type strains if given correctly.

Only a few CDV strains have been characterized in animal models. Among these, the R252 and A75/17 strains represent typical wild-type isolates that cause an acute disease of variable severity in dogs and result in a slow developing demyelinating leukoencephalomyelitis in a subset of animals (Appel, 1970; Krakowka and Koestner, 1977). In contrast, the Snyder Hill strain, which was originally developed by repeated passage in the central nervous system (CNS) of dogs to generate a virulent challenge strain for vaccine studies, causes a rapid, acute, and fatal polioencephalitis in a high proportion of infected animals (Gillespie and Rickard, 1956). More recently, the 5804P strain, obtained after one passage of a canine wild-type isolate in ferrets, has been used to investigate different aspects of Morbillivirus pathogenesis in this model (Pillet *et al.*, 2009).

4.3 Pathogenesis of Acute CDV

4.3.1 Virus receptors

Among the known Morbillivirus receptors, the signaling lymphocyte activation molecule (SLAM or CD150), which is part of the Ig superfamily, is found on activated lymphocytes and certain macrophage and dendritic cell subsets and explains the strong lymphotropism observed (Tatsuo *et al.*, 2001). CD9, a member of the tetraspan transmembrane protein family, may also play a role in CDV entry, but in contrast to SLAM, there is no evidence for a direct interaction with the virus, suggesting that it may act as part of a receptor complex or enhance the expression or activity of another still unknown receptor molecule (Schmid *et al.*, 2000; Singethan *et al.*, 2008). The adherens junction protein nectin-4 or poliovirus-receptor-like-4 protein (PVRL-4) was recently identified as a MeV epithelial cell receptor, and its expression in trachea, lung and other epithelial tissues overlaps with known sites of infection (Mühlebach *et al.*, 2011; Noyce *et al.*, 2011). The high conservation of nectin-4 molecules from different species and the involvement of corresponding amino acids in the CDV and MeV H

proteins in the interaction with nectin-4 strongly suggest that its receptor function may be conserved among all morbilliviruses (Langedijk *et al.*, 2011; Pratakpiriya *et al.*, 2012; Sawatsky *et al.*, 2012). However, the infection of endothelial and CNS cells observed in CDV-infected animals points towards the existence of additional receptor molecules.

4.3.2 Route of infection, tropism, spread

CDV is transmitted primarily via the respiratory route, especially inhalation of airborne viruses or infective aerosol droplets. However, virus can be found in any discharge or secretion (Shen and Gorham, 1978; Shen *et al.*, 1981). The initial replication occurs in the lymphoid tissues of the respiratory tract, including the tonsils (Krakowka *et al.*, 1980; Lemon *et al.*, 2011). Approximately 3 days post-infection, the virus spreads via infected immune cells through lymphatic and blood vessels, leading to a generalized infection of all lymphoid organs, including bone marrow, lymph nodes, spleen, thymus and tissue-associated lymphatic structures (Appel, 1970; von Messling *et al.*, 2004). Several days later, a second viremia results in high fever and spread to epithelial tissues throughout the body including the mucosa and skin (Appel, 1970; Blixenkrone-Moller, 1989; von Messling *et al.*, 2004). At the peak of infection, CDV-infected cells are also frequently found in the CNS (Summers *et al.*, 1979; Baumgärtner *et al.*, 1989; Rudd *et al.*, 2006). Histologically, these cells can often be identified by the presence of cytoplasmic and intranuclear inclusion bodies (Confer *et al.*, 1975). In non-lethal infections, a vigorous cellular and humoral immune response leads to virus clearance within 2–3 weeks (Appel *et al.*, 1982; von Messling *et al.*, 2003). However, in some cases persistence is observed in the CNS, footpads and the uvea (Summers *et al.*, 1983; Gröne *et al.*, 2003).

4.3.3 Clinical disease

First clinical signs, including lethargy, anorexia, weight loss, leukopenia and fever, are observed at the onset of viremia (Coffin and Liu, 1957; Appel, 1970; von Messling *et al.*, 2003). Serous nasal and ocular discharge, respiratory, gastrointestinal and occasionally nervous signs, as well as a cutaneous rash (Fig. 4.2A), characterizing the acute systemic disease phase, coincide with dissemination to epithelial tissues (Fig. 4.2B). In addition to serous to mucopurulent

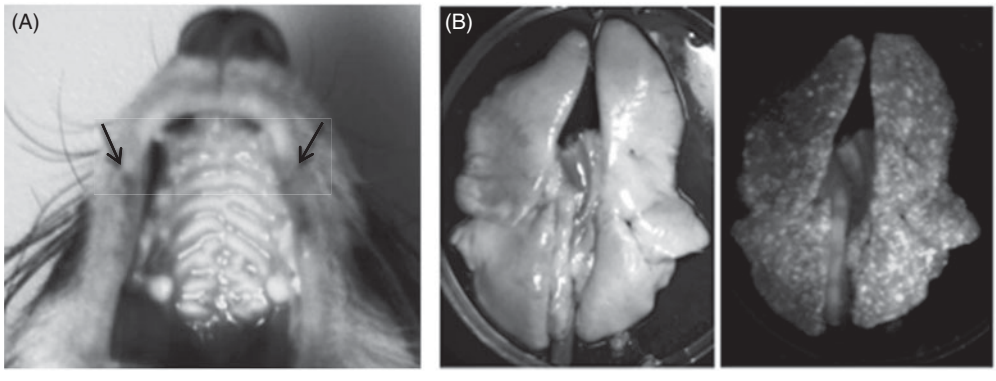

Fig. 4.2. Macroscopic visualization of CDV infection. (A) Overlay picture of the mouth of an infected animal showing characteristic rash on the lips and mucosal surfaces. (B) Normal light and eGFP photographs of the lung at 14 days after infection.

rhinitis, interstitial pneumonia, necrotizing bronchiolitis and, in cases of secondary bacterial infection, suppurative bronchopneumonia, dominate the respiratory manifestations (Appel, 1969; Caswell and Williams, 2007). A catarrhal enteritis with depletion of Peyer's patches and a characteristic pustular dermatitis (distemper exanthema), most prominent at the thighs, ventral abdomen and the inner surface of the ear pinnae, are also frequently observed (Maeda *et al.*, 1994; von Messling *et al.*, 2004). In cases with CNS involvement, various neurological signs including ataxia, myoclonus, nystagmus, postural reaction deficits and tetraparesis or -plegia can also occur (Koutinas *et al.*, 2002). Neurological signs in dogs tend to be progressive, and surviving dogs may show lifelong residual signs such as persistent myoclonus (Tipold, 1995).

Animals that contract the disease during their growing phase develop a characteristic enamel hypoplasia because of infection of the growing tooth buds and ameloblasts during tooth development (Dubielzig *et al.*, 1981; Bittegeko *et al.*, 1995). Young dogs can also suffer from persistence of the primary spongiosa in the metaphyses of long bones, also referred to as metaphyseal osteosclerosis of the growth retardation lattice, which results from a necrosis of CDV-infected osteoclasts (Baumgärtner *et al.*, 1995). In rare cases, infection can lead to development of hard pad disease, a progressive hyperkeratosis of the footpads and nasal planum (Gröne *et al.*, 2003).

4.3.4 Immunosuppression

CDV infection causes a severe acute immunosuppression, which delays clearance of the infection and predisposes the animal to opportunistic secondary infections (Krakowka *et al.*, 1975; von Messling *et al.*, 2003). The dramatic loss of immune cells during early infection stages results in generalized depletion of all lymphoid tissues, lymphadenopathy and reduced thymus size (Krakowka and Koestner, 1977; Iwatsuki *et al.*, 1995). The bone marrow is generally only mildly affected and mainly shows non-specific changes, including necrosis of hematopoietic cells or hypercellularity and granulocytic hyperplasia (Breuer *et al.*, 1998). In addition to the direct virus-induced death of infected immune cells, an important depletion of uninfected lymphocytes is also observed (Pillet and von Messling, 2009). Different mechanisms have been proposed, including a contact-mediated growth arrest or Fas-mediated activation-induced apoptosis of lymphoid cells (Niewiesk *et al.*, 1999; Moro *et al.*, 2003; Schobesberger *et al.*, 2005).

To counteract the inhibitory effect of the innate immune response, viruses have evolved mechanisms to interfere with the IFN-signaling cascade. In the case of morbilliviruses, the accessory V protein acts as the primary innate immune-response interference protein (Palosaari *et al.*, 2003). In addition to preventing the nuclear translocation of STAT molecules, thereby disrupting downstream signaling, V also blocks mda5-mediated activation of innate immune responses (Röthlisberger *et al.*, 2010; Ramachandran and Horvath, 2010). While the contribution of the different IFN-signaling pathways to morbillivirus pathogenesis remains to be characterized, the extent of the overall inhibition of innate immune activation correlates with disease severity (Svitek and von Messling, 2007).

Most of the infected immune cells in non-immune tissues are resident macrophages and dendritic cells (Iwatsuki *et al.*, 1995; Wünschmann *et al.*, 2000), suggesting a possible interference of the virus with antigen presentation and other aspects of the adaptive immune response. The detection of lower IL-1 expression levels and increased production of prostaglandin E2 in monocytes from infected animals (Krakowka *et al.*, 1987b), and of defects in the formation of germinal centres and lower IgG antibody levels in persistently infected dogs (Winters *et al.*, 1983), support this hypothesis, but a more detailed characterization of the effects and underlying mechanisms is necessary to understand viral interference with the adaptive immune response. Animals that survive the disease develop robust cellular and humoral immune

responses that confer lifelong protection (Schultz *et al.*, 2010), illustrating that the viral interference with the host response will ultimately be overcome.

4.4 CNS Complications

4.4.1 Virus entry in the CNS

In addition to its tropism for immune and epithelial cells, CDV frequently infects the CNS (Baumgärtner *et al.*, 1989). Neurological complications have been reported in up to 30% of dogs, and occur even more frequently in wild carnivores (Summers *et al.*, 1984; Griot *et al.*, 2003). If the animal does not die during the acute disease, the CNS infection frequently progresses to a chronic demyelinating leukoencephalitis (Beineke *et al.*, 2009). Because of this high incidence of CNS infection, CDV is often used to study morbillivirus neuroinvasion.

CNS entry via haematogenous, cerebrospinal and neural routes has been documented. Infected immune cells may cross the blood–brain barrier or lead to infection of capillary endothelial cells, thereby giving the virus direct access to the CNS (Krakowka *et al.*, 1987a; Krakowka, 1989). CDV antigen is detected in CNS capillaries and venular endothelia at 5–6 days post-infection and is found a few days later in adjacent pericytes, astrocytic foot processes and meningeal cap cells (Axthelm and Krakowka, 1987; Rudd *et al.*, 2006). From there, the infection spreads to adjacent neurons or pia mater if it is not contained by the developing immune system (Summers *et al.*, 1979; Axthelm and Krakowka, 1987).

The choroid plexus (CP) represents a highly vascularized structure within the ventricular system of the brain, which is covered by epithelial cells and produces the cerebrospinal fluid (CSF). A productive CDV infection of CP epithelium is usually detected around 10 days post-infection, when the virus spreads to epithelial tissues throughout the body, creating an intracranial source of free infectious virus (Higgins *et al.*, 1982; Rudd *et al.*, 2006). Thereafter, the continuous flow of CSF brings the virus in contact with adjacent ependymal cells and subependymal white matter (Higgins *et al.*, 1982; Ludlow *et al.*, 2012). The periventricular and subpial location of many demyelinating lesions indicates a prominent role of this cerebrospinal pathway in CDV CNS infection (Vandevelde *et al.*, 1985).

Even though the CNS is considered an immune privileged site because of the blood–brain barrier, several cranial nerves are in direct contact with mucosal epithelia of the upper respiratory tract. Anterograde spread from respiratory mucosa along cranial nerves into the CNS has been documented for several neurotropic viruses, including herpes and coronaviruses (Barthold, 1988; Mori *et al.*, 2005), and a study using an eGFP-expressing neurovirulent CDV strain first demonstrated neuroinvasion via the olfactory nerve in experimentally infected ferrets (Rudd *et al.*, 2006). The observed concentration of infection in the olfactory bulb, cerebellum, hippocampus and especially around cranial nuclei in the brainstem indicates that transneural invasion via cranial nerves plays an important role in CDV CNS invasion (Rudd *et al.*, 2006; Ludlow *et al.*, 2012).

4.4.2 CDV target cells in the CNS

After entering the CNS, CDV efficiently infects astrocytes and neurons (Rudd *et al.*, 2006). Astrocytes represent 95% of all infected cells in early non-inflammatory lesions (Mutinelli *et al.*, 1989), and immature vimentin-positive astrocyte-like cells found in advanced lesions show an even higher susceptibility to CDV than mature GFAP-positive astrocytes (Seehusen *et al.*, 2007). Neurons are predominantly infected in the early phase of distemper encephalitis, and often express a disproportionally low amount of viral protein compared with the mRNA present (Nesseler *et al.*, 1999), reproducing findings described for subacute sclerosing panencephalitis, a rare MeV complication (Nesseler *et al.*, 1997). Despite the characteristic demyelination of distemper lesions, only few (McCullough *et al.*, 1974; Blakemore *et al.*,

1989) or no infected oligodendrocytes were found in various older studies (Zurbriggen and Vandevelde, 1983; Zurbriggen *et al.*, 1986). More recent work revealed that this low detection rate is the result of restricted replication in these cells, since a presence of CDV nucleic acids but a lack of viral antigen were observed (Zurbriggen *et al.*, 1993). Similar to astrocytes, the susceptibility of oligodendrocytes seems to depend on the stage of cellular differentiation (Imbschweiler *et al.*, 2012). While multipolar mature oligodendrocytes are only rarely infected, a subset of bipolar oligodendrocyte precursor cells expressing galactocerebroside show a high susceptibility for some CDV strains, at least *in vitro* (Pearce-Kelling *et al.*, 1991). The CDV-infection rate of microglia is still unknown. However, these resident immune cells have been implicated in the activation of the local immune response in acute distemper encephalitis (Rudd *et al.*, 2010).

4.4.3 Pathology of nervous distemper lesions

CDV CNS lesions are categorized depending on their pathomorphology as acute, subacute, chronic and sclerotic plaques (Summers and Appel, 1987; Higgins *et al.*, 1982). Different plaque types can occur simultaneously in the brain of affected dogs and can be found in the white and grey matter (Wünschmann *et al.*, 1999; Baumgärtner *et al.*, 1989). Initially, the virus targets protoplasmatic astrocytes and neurons in cortical areas and brain-stem nuclei, resulting in a polioencephalitis (Nesseler *et al.*, 1999). Histologically, these grey matter lesions are characterized by neuronal necrosis, neuronophagia by microglia/macrophages and T cells, perivascular cuffing of monocytic inflammatory cells and intranuclear inclusion bodies in neurons and astrocytes (Nesseler *et al.*, 1999; Fig. 4.3A and B).

As early as 4 weeks after infection, a focal vacuolization of the subependymal white matter due to an intramyelin oedema, together with few activated astrocytes and macrophages/microglia can be found adjacent to the ventricles (Summers and Appel, 1994). At later stages, virus is found in the cerebellar and less frequently cerebral and spinal cord white matter, triggering a demyelinating leukoencephalitis (Baumgärtner *et al.*, 1989; Fig. 4.3C and D). Finally, the destroyed tissue in chronic lesions is replaced by astrocytic scars, which show only limited remyelination of denuded axons (Vandevelde *et al.*, 1982a; Summers and Appel, 1987).

Despite the abundance of CDV antigen, morphological signs of inflammation are rare in acute lesions, most likely due to the strong systemic immunosuppression (Baumgärtner *et al.*, 1989; Alldinger *et al.*, 1993). Nevertheless, numerous T cells are found in acute demyelinating lesions, which are most likely attracted by chemokines secreted from activated microglia (Tipold *et al.*, 1999). Concurrently with the recovery and repopulation of the lymphoid organs, infiltration of the CNS by mononuclear cells increases, resulting in restriction of virus replication but also in increased immunopathology (Summers and Appel, 1994; Wünschmann *et al.*, 1999).

4.4.4 Antiviral immune response and immunopathology in the CNS

The early phase of distemper encephalitis is characterized by a minimal influx of inflammatory cells because of the general immunosuppression. Consequently, resident CNS immune cells play the main role in the initial response by releasing a vast array of cytokines and chemokines (Tipold *et al.*, 1999). Type I IFN was detected in the serum and CSF of CDV-infected dogs at 4 and 5 days post-infection, respectively (Tsai *et al.*, 1982), and their detection in the brains of these animals for up to 56 days post-infection even prompted the authors to suggest type I IFN expression as a marker for CDV persistence. A subsequent investigation of the immune response in the CNS during CDV infection revealed a prominent up-regulation of the pro-inflammatory cytokines IL-6, IL-8, IL-12 and TNF-α and no change in the expression of the immunomodulatory cytokines IL-10 and TGF-β in the cerebella of distemper dogs

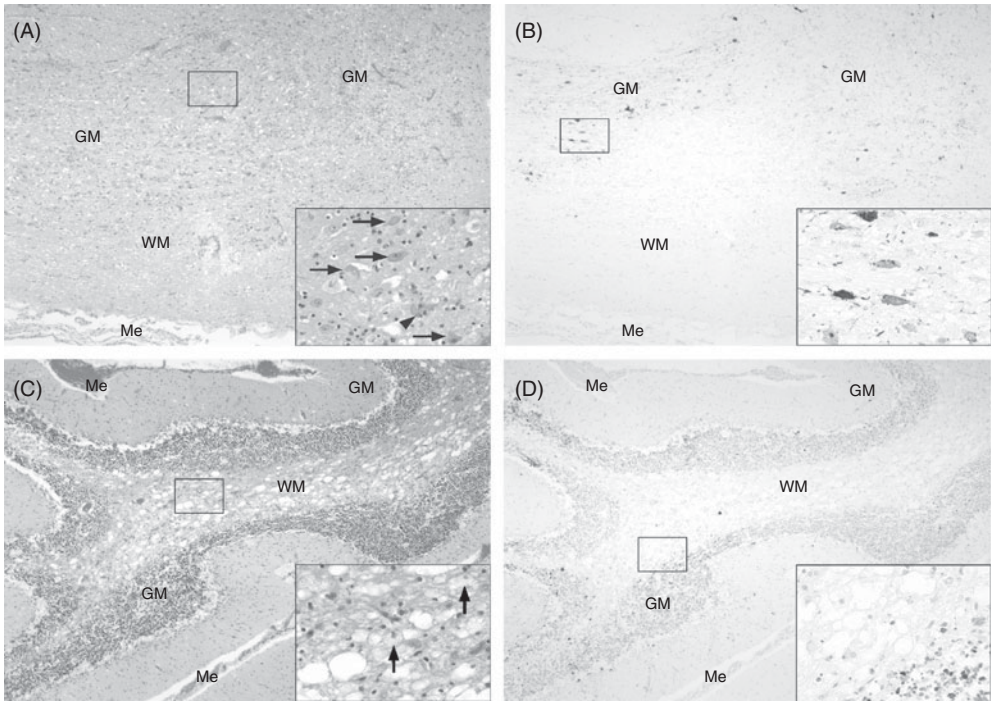

Fig. 4.3. Distemper lesions in the canine CNS. (A and B) Medulla oblongata. Mild polioencephalitis with microglia activation and neuronal necrosis (arrows). One necrotic neuron shows an intranuclear inclusion body and neuronophagia (arrowhead). (C and D) Cerebellum. Mild leukoencephalitis with severe vacuolation of the white matter. Note the myelin-laden microglia/macrophages (gitter cells) in the higher magnification (arrows). CDV antigen can be found predominantly in the grey matter (GM). Only few infected cells are present in the white matter (WM). Meninx (Me). Haematoxylin and eosin staining (A and C). Immunohistochemistry with mouse anti-CDV antibody, diaminobenzidine and haematoxylin counterstain (B and D).

(Markus *et al.*, 2002). Together, these signaling proteins activate the innate antiviral response, induce the up-regulation of major histocompatibility complex (MHC) class II molecules necessary for antigen presentation and attract additional immune cells (Alldinger *et al.*, 1996).

Immunohistochemical staining of white matter lesions revealed the presence of IL-1β and IL-6 in $CD3^+$ T-cells and macrophages/microglia in early and chronic plaques, while astrocytes especially in acute lesions expressed mainly TNF-α, and IL-12 was found in individual perivascular cells in the vicinity of chronic plaques (Gröne *et al.*, 2000). The release of proteolytic enzymes, increased phagocytic activity and oxygen radical production by stimulated macrophages/microglia not only contribute to infection control but also result in damage to the neighbouring parenchyma, including myelin sheaths (Cammer *et al.*, 1978; Stein *et al.*, 2004), hence its name 'bystander demyelination' (Brügger *et al.*, 1992).

In the late phase, starting at roughly 7 weeks post-infection, abundant blood-borne immune cells invade the preexisting white matter lesions because of the recovery and repopulation of peripheral lymphoid organs (Wünschmann *et al.*, 1999). The resulting intralesional accumulation of $CD4^+$ T cells, B cells and plasma cells, leads to a strong local production of antiviral antibodies, which can be easily detected in the CSF (Vandevelde *et al.*, 1982b). The following intense inflammatory reaction causes a dramatic reduction of CDV antigen in the CNS but often fails to eliminate the virus completely. In addition,

the interaction of CDV–immune complexes with the complement system can trigger a complement-dependent antibody-mediated cytotoxicity aggravating demyelination (Vandevelde *et al.*, 1986). The detection of antiviral antibodies bound to the surface of infected CNS cells by the Fc receptors on neighbouring macrophages can trigger the release of reactive oxygen species contributing further to oligodendroglial degeneration (Bürge *et al.*, 1989; Griot *et al.*, 1989). Finally, an antibody-dependent T-cell-mediated cytotoxicity to oligodendrocytes may enhance myelin loss (Vandevelde *et al.*, 1982b; Botteron *et al.*, 1992).

In addition to the immunopathological mechanisms described so far, which rely on the ongoing presence of viral antigen in white matter lesions, an autoimmune response against host myelin may also contribute to the observed demyelination. The destruction of oligodendrocytes as 'innocent bystanders' unquestionably liberates abundant previously hidden myelin antigens, which can activate autoreactive T cells and generate anti-myelin-specific antibodies (Krakowka *et al.*, 1973; Wünschmann *et al.*, 2000). Moreover, antibodies directed against different myelin epitopes have been detected in the CSF (Krakowka *et al.*, 1973; Rima *et al.*, 1991). However, the relevance of these myelin-specific autoimmune reactions remains unknown because of the presence of abundant anti-myelin antibodies in dogs with resolving lesions (Vandevelde *et al.*, 1986).

4.4.5 Mechanisms of viral CNS persistence

CNS persistence represents an important aspect of the CDV disease spectrum, which remains poorly understood. There are however several indications that the virus evades immune recognition and subsequent clearance by limiting antigen presentation through specific interference with viral protein synthesis despite abundant expression of all viral mRNAs (Nesseler *et al.*, 1997, 1999). Furthermore, CDV spreads almost exclusively by cell–cell fusion, preventing the contact of tissue-resident immune cells with viral particles (Müller *et al.*, 1995). Because of the low expression levels of these fusogenic complexes on the cell surface, cell–cell spread is restricted to a few cells surrounding an infected one (Plattet *et al.*, 2005; Wyss-Fluehmann *et al.*, 2010). Finally, the detection of viral particles in intact axons in combination with evidence of an anterograde infection of the CNS via the olfactory route, provide convincing evidence of a trans-synaptic interneuronal spread, which may facilitate dissemination to distant CNS structures along neuronal signaling pathways, thereby escaping local immune detection (Higgins *et al.*, 1982; Rudd *et al.*, 2006).

4.5 Conclusions

CDV remains one of the most important infectious diseases in domestic dogs and many other domestic and wild carnivores, with outbreaks occurring worldwide. Its broad host range and species-dependent differences in disease severity make CDV an attractive system for the identification of host range determinants as well as an ongoing challenge for wildlife conservation efforts. In addition, the similarities in the clinical course of CDV and MeV lead to its increasing use for Morbillivirus pathogenesis studies and the development of new vaccines and antivirals.

References

Alldinger, S., Baumgärtner, W. and Orvell, C. (1993) Restricted expression of viral surface proteins in canine distemper encephalitis. *Acta Neuropathologica* 85, 635–645.

Alldinger, S., Wunschmann, A., Baumgärtner, W., Voss, C. and Kremmer, E. (1996) Up-regulation of major histocompatibility complex class II antigen expression in the central nervous system of dogs with spontaneous canine distemper virus encephalitis. *Acta Neuropathologica* 92, 273–280.

Appel, M.J. (1969) Pathogenesis of canine distemper. *American Journal of Veterinary Research* 30, 1167–1182.

Appel, M.J. (1970) Distemper pathogenesis in dogs. *Journal of the American Veterinary Medical Association* 156, 1681–1684.

Appel, M.J., Shek, W.R. and Summers, B.A. (1982) Lymphocyte-mediated immune cytotoxicity in dogs infected with virulent canine distemper virus. *Infection and Immunity* 37, 592–600.

Appel, M.J., Reggiardo, C., Summers, B.A., Pearce-Kelling, S., Maré, C.J., Noon, T.H., Reed, R.E., Shivly, J.N. and Orvell, C. (1991) Canine distemper virus infection and encephalitis in javelinas (collared peccaries). *Archives of Virology* 119, 147–152.

Axthelm, M.K. and Krakowka, S. (1987) Canine distemper virus: the early blood–brain barrier lesion. *Acta Neuropathologica* 75, 27–33.

Barthold, S.W. (1988) Olfactory neural pathway in mouse hepatitis virus nasoencephalitis. *Acta Neuropathologica* 76, 502–506.

Baumgärtner, W., Orvell, C. and Reinacher, M. (1989) Naturally occurring canine distemper virus encephalitis: distribution and expression of viral polypeptides in nervous tissues. *Acta Neuropathologica* 78, 504–512.

Baumgärtner, W., Boyce, R.W., Weisbrode, S.E., Aldinger, S., Axthelm, M.K. and Krakowka, S. (1995) Histologic and immunocytochemical characterization of canine distemper-associated metaphyseal bone lesions in young dogs following experimental infection. *Veterinary Pathology* 32, 702–709.

Beineke, A., Puff, C., Seehusen, F. and Baumgärtner, W. (2009) Pathogenesis and immunopathology of systemic and nervous canine distemper. *Veterinary Immunology and Immunopathology* 127, 1–18.

Bittegeko, S.B., Arnbjerg, J., Nkya, R. and Tevik, A. (1995) Multiple dental developmental abnormalities following canine distemper infection. *Journal of the American Animal Hospital Association* 31, 42–45.

Blakemore, W.F., Summers, B.A. and Appel, M.G. (1989) Evidence of oligodendrocyte infection and degeneration in canine distemper encephalomyelitis. *Acta Neuropathologica* 77, 550–553.

Blixenkrone-Møller, M. (1989) Detection of intracellular canine distemper virus antigen in mink inoculated with an attenuated or a virulent strain of canine distemper virus. *American Journal of Veterinary Research* 50, 1616–1620.

Bolt, G., Jensen, T.D., Gottschalck, E., Arctander, P., Appel, M.J., Buckland, R. and Blixenkrone-Møller, M. (1997) Genetic diversity of the attachment (H) protein gene of current field isolates of canine distemper virus. *Journal of General Virology* 78, 367–372.

Botteron, C., Zurbriggen, A., Griot, C. and Vandevelde, M. (1992) Canine distemper virus-immune complexes induce bystander degeneration of oligodendrocytes. *Acta Neuropathologica* 83, 402–407.

Breuer, W., Stahr, K., Majzoub, M. and Hermanns, W. (1998) Bone-marrow changes in infectious diseases and lymphohaemopoietic neoplasias in dogs and cats – a retrospective study. *Journal of Comparative Pathology* 119, 57–66.

Brügger, M., Jungi, T.W., Zurbriggen, A. and Vandevelde, M. (1992) Canine distemper virus increases procoagulant activity of macrophages. *Virology* 190, 616–623.

Bürge, T., Griot. C., Bandevelde, M. and Peterhans, E. (1989) Antiviral antibodies stimulate production of reactive oxygen species in cultured canine brain cells infected with canine distemper virus. *Journal of Virology* 63, 2790–2797.

Cammer, W., Bloom, B.R., Norton, W.T. and Gordon, S. (1978) Degradation of basic protein in myelin by neutral proteases secreted by stimulated macrophages: a possible mechanism of inflammatory demyelination. *Proceedings of the National Academy of Sciences USA* 75, 1554–1558.

Carré, H. (1905) Sur la maladie des jeunes chiens. *Comptes Rendus de l'Académie des Sciences* 140, 689–690.

Caswell, J.L. and Williams, K.J. (2007) Respiratory system. In Maxie, M.G. (ed.) *Jubb, Kennedy & Palmer's Pathology of Domestic Animals*. Saunders Elsevier, Edinburgh, pp. 635–636.

Coffin, D.L. and Liu, C. (1957) Studies of canine distemper infection by means of fluorescein-labeled antibody. II. The pathology and diagnosis of the naturally occurring disease in dogs and the antigenic nature of the inclusion body. *Virology* 3, 132–145.

Confer, A.W., Kahn, D.E., Koestner, A. and Krakowka, S. (1975) Biological properties of a canine distemper virus isolate associated with demyelinating encephalomyelitis. *Infection and Immunity* 11, 835–844.

Deem, S.L., Spelman, L.H., Yates, R.A. and Montali, R.J. (2000) Canine distemper in terrestrial carnivores: a review. *Journal of Zoo and Wildlife Medicine* 31, 441–451.

Demeter, Z., Palade, E.A., Hornyák, A. and Rusvai, M. (2010) Controversial results of the genetic analysis of a canine distemper vaccine strain. *Veterinary Microbiology* 142, 420–426.

Diallo, A. (1990) Morbillivirus group: genome organisation and proteins. *Veterinary Microbiology* 23, 155–163.

Dubielzig, R.R., Higgins, R.J. and Krakowka, S. (1981) Lesions of the enamel organ of developing dog teeth following experimental inoculation of gnotobiotic puppies with canine distemper virus. *Veterinary Pathology* 18, 684–689.

Gámiz, C., Martella, V., Ulloa, R., Fajardo, R., Quijano-Hernandéz, I. and Martínez, S. (2011) Identification of a new genotype of canine distemper virus circulating in America. *Veterinary Research Communications* 35, 381–390.

Gillespie, J.H. and Rickard, C.G. (1956) Encephalitis in dogs produced by distemper virus. *American Journal of Veterinary Research* 17, 103–108.

Griot, C., Bürge, T., Vandevelde, M. and Peterhans, E. (1989) Antibody-induced generation of reactive oxygen radicals by brain macrophages in canine distemper encephalitis: a mechanism for bystander demyelination. *Acta Neuropathologica* 78, 396–403.

Griot, C., Vandevelde, M., Schobesberger, M. and Zurbriggen, A. (2003) Canine distemper, a re-emerging morbillivirus with complex neuropathogenic mechanisms. *Animal Health Research Reviews* 4, 1–10.

Gröne, A., Alldinger, S. and Baumgärtner, W. (2000) Interleukin-1beta, -6, -12 and tumor necrosis factor-alpha expression in brains of dogs with canine distemper virus infection. *Journal of Neuroimmunology* 110, 20–30.

Gröne, A., Groeters, S., Koutinas, A., Saridomichelakis, M. and Baumgärtner, W. (2003) Non-cytocidal infection of keratinocytes by canine distemper virus in the so-called hard pad disease of canine distemper. *Veterinary Microbiology* 96, 157–163.

Haig, D.A. (1956) Canine distemper immunization with avianized virus. *Ondertepoort Journal of Veterinary Research* 27, 19–53.

Hall, A.J. (1995) Morbilliviruses in marine mammals. *Trends in Microbiology* 3, 4–9.

Higgins, R.J., Krakowka, S.G., Metzler, A.E. and Koestner, A. (1982) Experimental canine distemper encephalomyelitis in neonatal gnotobiotic dogs. A sequential ultrastructural study. *Acta Neuropathologica* 57, 287–295.

Imbschweiler, I., Seehusen, F., Peck, C.T., Omar, M., Baumgärtner, W. and Wewetzer, K. (2012) Increased p75 neurotrophin receptor expression in the canine distemper virus model of multiple sclerosis identifies aldynoglial Schwann cells that emerge in response to axonal damage. *Glia* 60, 358–371.

Iwasaki, M., Takeda, M., Shirogane, Y., Nakatsu, Y., Nakamura, T. and Yanagi, Y. (2009) The matrix protein of measles virus regulates viral RNA synthesis and assembly by interacting with the nucleocapsid protein. *Journal of Virology* 83, 10,374–10,383.

Iwatsuki, K., Okita, M., Ochikubo, F., Gemma, T., Shin, Y.S., Miyashita, N., Mikami, T. and Kai, C. (1995) Immunohistochemical analysis of the lymphoid organs of dogs naturally infected with canine distemper virus. *Journal of Comparative Pathology* 113, 185–190.

Kennedy, S., Kuiken, T., Jepson, P.D., Deaville, R., Forsyth, M., Barrett, T., van de Bildt, M.W., Osterhaus, A.D., Eybatov, T., Duck, C., Kydyrmanov, A., Mitrofanov, I. and Wilson, S. (2000) Mass die-off of Caspian seals caused by canine distemper virus. *Emerging Infectious Diseases* 6, 637–639.

Koutinas, A.F., Polizopoulou, Z.S., Baumgärtner, W., Lekkas, S. and Kontos, V. (2002) Relation of clinical signs to pathological changes in 19 cases of canine distemper encephalomyelitis. *Journal of Comparative Pathology* 126, 47–56.

Krakowka, S. (1989) Canine distemper virus infectivity of various blood fractions for central nervous system vasculature. *Journal of Neuroimmunology* 21, 75–80.

Krakowka, S. and Koestner, A. (1977) Comparison of canine distemper virus strains in gnotobiotic dogs: effects on lymphoid tissues. *American Journal of Veterinary Research* 38, 1919–1922.

Krakowka, S., McCullough, B., Koestner, A. and Olson, R. (1973) Myelin-specific autoantibodies associated with central nervous system demyelination in canine distemper virus infection. *Infection and Immunity* 8, 819–827.

Krakowka, S., Cockerell, G. and Koestner, A. (1975) Effects of canine distemper virus infection on lymphoid function in vitro and in vivo. *Infection and Immunity* 11, 1069–1078.

Krakowka, S., Higgins, R.J. and Koestner, A. (1980) Canine distemper virus: review of structural and functional modulations in lymphoid tissues. *American Journal of Veterinary Research* 41, 284–292.

Krakowka, S., Ringler, S.S., Lewis, M., Olsen, R.G. and Axthelm, M.K. (1987a) Immunosuppression by canine distemper virus: modulation of in vitro immunoglobulin synthesis, interleukin release and prostaglandin E2 production. *Veterinary Immunology and Immunopathology* 15, 181–201.

Krakowka, S., Cork, S.L., Winkelstein, J.A. and Axthelm, M.K. (1987b) Establishment of central nervous system infection by canine distemper virus: breach of the blood–brain barrier and facilitation by antiviral antibody. *Veterinary Immunology and Immunopathology* 17, 471–482.

Lamb, R.A. and Parks, G.D. (2007) Paramyxoviridae: the viruses and their replication. In: Knipe, D.M. and Howley, P.M. (eds) *Fields Virology*. Lippincott Williams & Wilkins, Philadelphia, Pennsylvania, pp. 1449–1498.

Langedijk, J.P., Janda, J., Origgi, F.C., Örvell, C., Vandevelde, M., Zurbriggen, A. and Plattet, P. (2011) Canine distemper virus infects canine keratinocytes and immune cells by using overlapping and distinct regions located on one side of the attachment protein. *Journal of Virology* 85, 11,242–11,254.

Lemon, K., de Vries, R.D., Mesman, A.W., McQuaid, S., van Amerongen, G., Yüksel, S., Ludlow, M., Rennick, L.J., Kuiken, T., Rima, B.K., Geijtenbeek, T.B., Osterhaus, A.D., Duprex, W.P. and de Swart, R.L. (2011) Early target cells of measles virus after aerosol infection of non-human primates. *PLoS Pathogens* 7, e1001263.

Ludlow, M., Nguyen, D.T., Silin, D., Lyubomska, O., de Vries, R.D., von Messling, V., McQuaid, S., de Swart, R.L. and Duprex, W.P. (2012) Recombinant canine distemper virus strain Snyder Hill expressing green or red fluorescent proteins causes meningoencephalitis in the ferret. *Journal of Virology* 86, 7508–7519.

Maeda, H., Ozaki, K., Takaqi, Y., Sawashima, K. and Narama, I. (1994) Distemper skin lesions in a dog. *Zentralblatt der Veterinarmedizin A* 41, 247–250.

Markus, S., Failing, K. and Baumgärtner, W. (2002) Increased expression of pro-inflammatory cytokines and lack of up-regulation of anti-inflammatory cytokines in early distemper CNS lesions. *Journal of Neuroimmunology* 125, 30–41.

McCullough, B., Krakowka, S. and Koestner, A. (1974) Experimental canine distemper virus-induced demyelination. *Laboratory Investigations* 31, 216–222.

Mori, I., Goshima, F., Ito, H., Koide, N., Yoshida, T., Yokochi, T, Kimura, Y. and Nishiyama, Y. (2005) The vomeronasal chemosensory system as a route of neuroinvasion by herpes simplex virus. *Virology* 334, 51–58.

Moro, L., de Sousa Martins, A., de Moraes Alves, C., de Araújo Santos, F.G., dos Santos Nunes, J.E., Carneiro, R.A., Carvalho, R. and Vasconcelos, A.C. (2003) Apoptosis in canine distemper. *Archives of Virology* 148, 153–164.

Mühlebach, M.D., Mateo, M., Sinn, P.L., Prüfer, S., Uhlig, K.M., Leonard, V.H., Navaratnarajah, C.K., Frenzke, M., Wong, X.X., Sawatsky, B., Ramachandran, S., McCray, P.B. Jr, Cichutek, K., von Messling, V., Lopez, M. and Cattaneo, R. (2011) Adherens junction protein nectin-4 is the epithelial receptor for measles virus. *Nature* 480, 530–533.

Müller, C.F., Fatzer, R.S., Beck, K., Vandevelde, M. and Zurbriggen, A. (1995) Studies on canine distemper virus persistence in the central nervous system. *Acta Neuropathologica* 89, 438–445.

Mutinelli, F., Vandevelde, M., Griot, C. and Richard, A. (1989) Astrocytic infection in canine distemper virus-induced demyelination. *Acta Neuropathologica* 77, 333–335.

Nesseler, A., Baumgärtner, W., Gaedke, K. and Zurbriggen, A. (1997) Abundant expression of viral nucleoprotein mRNA and restricted translation of the corresponding viral protein in inclusion body polioencephalitis of canine distemper. *Journal of Comparative Pathology* 116, 291–301.

Nesseler, A., Baumgärtner, W., Zurbriggen, A. and Orvell, C. (1999) Restricted virus protein translation in canine distemper virus inclusion body polioencephalitis. *Veterinary Microbiology* 69, 23–28.

Niewiesk, S., Ohnimus, H., Schnorr, J.J., Götzelmann, M., Schneider-Schaulies, S., Jassoy, C. and ter Meulen, V. (1999) Measles virus-induced immunosuppression in cotton rats is associated with cell cycle retardation in uninfected lymphocytes. *Journal of General Virology* 80, 2023–2029.

Noyce, R.S., Bondre, D.G., Ha, M.N., Lin, L.T., Sisson, G., Tsao, M.S. and Richardson, C.D. (2011) Tumor cell marker PVRL4 (nectin 4) is an epithelial cell receptor for measles virus. *PLoS Pathogens* 7, e1002240.

Palosaari, H., Parisien, J.P., Rodriguez, J.J., Ulane, C.M. and Horvath, C.M. (2003) STAT protein interference and suppression of cytokine signal transduction by measles virus V protein. *Journal of Virology* 77, 7635–7644.

Pearce-Kelling, S., Mitchell, W.J., Summers, B.A. and Appel, M.J. (1991) Virulent and attenuated canine distemper virus infects multiple dog brain cell types in vitro. *Glia* 4, 408–416.

Pillet, S. and von Messling, V. (2009) Canine distemper virus selectively inhibits apoptosis progression in infected immune cells. *Journal of Virology* 83, 6279–6287.

Pillet, S., Svitek, N. and von Messling, V. (2009) Ferrets as a model for morbillivirus pathogenesis, complications, and vaccines. *Current Topics in Microbiology and Immunology* 330, 73–87.

Plattet, P., Rivals, J.P., Zuber, B., Brunner, J.M., Zurbriggen, A. and Wittek, R. (2005) The fusion protein of wild-type canine distemper virus is a major determinant of persistent infection. *Virology* 337, 312–326.

Pratakpiriya, W., Seki, F., Otsuki, N., Sakai, K., Fukuhara, H., Katamoto, H., Hirai, T., Maenaka, K., Techangamsuwan, S., Lan, N.T., Takeda, M. and Yamaguchi, R. (2012) Nectin4 is an epithelial cell receptor for canine distemper virus and involved in the neurovirulence. *Journal of Virology* 86, 10,207–10,210.

Pringle, C.R. (1997) The order Mononegavirales – current status. *Archives of Virology* 142, 2321–2326.

Qiu, W., Zheng, Y., Zhang, S., Fan, Q., Liu, G., Zhang, F., Wang, W., Liao, G. and Hu, R. (2011) Canine distemper outbreak in rhesus monkeys, China. *Emerging Infectious Diseases* 17, 1541–1543.

Ramachandran, A. and Horvath, C.M. (2010) Dissociation of paramyxovirus interferon evasion activities: universal and virus-specific requirements for conserved V protein amino acids in MDA5 interference. *Journal of Virology* 84, 11,152–11,163.

Rima, B.K., Duffy, N., Mitchell, W.J., Summers, B.A. and Appel, M.J. (1991) Correlation between humoral immune responses and presence of virus in the CNS in dogs experimentally infected with canine distemper virus. *Archives of Virology* 121, 1–8.

Robertshaw, D. (2010) Credit to Plowright for rinderpest eradication. *Science* 330, 1477.

Rockborn, G. (1959) An attenuated strain of canine distemper virus in tissue culture. *Nature* 184, 822.

Röthlisberger, A., Wiener, D., Schweizer, M., Peterhans, E., Zurbriggen, A. and Plattet, P. (2010) Two domains of the V protein of virulent canine distemper virus selectively inhibit STAT1 and STAT2 nuclear import. *Journal of Virology* 84, 6328–6343.

Rudd, P.A., Cattaneo, R. and von Messling, V. (2006) Canine distemper virus uses both the anterograde and the hematogenous pathway for neuroinvasion. *Journal of Virology* 80, 9361–9370.

Rudd, P.A., Bastien-Hamel, L.E. and von Messling, V. (2010) Acute canine distemper encephalitis is associated with rapid neuronal loss and local immune activation. *Journal of General Virology* 91, 980–989.

Saliki, J.T., Cooper, E.J. and Gustavson, J.P. (2002) Emerging morbillivirus infections of marine mammals: development of two diagnostic approaches. *Annals of the NY Academy of Sciences* 969, 51–59.

Sawatsky, B., Wong, X.X., Hinkelmann, S., Cattaneo, R. and von Messling, V. (2012) Canine distemper virus epithelial cell infection is required for clinical disease but not for immunosuppression. *Journal of Virology* 86, 3658–3666.

Schmid, E., Zurbriggen, A., Gassen, U., Rima, B., ter Meulen, V. and Schneider-Schaulies, J. (2000) Antibodies to CD9, a tetraspan transmembrane protein, inhibit canine distemper virus-induced cell–cell fusion but not virus-cell fusion. *Journal of Virology* 74, 7554–7561.

Schobesberger, M., Summerfield, A., Doherr, M.G., Zurbriggen, A. and Griot, C. (2005) Canine distemper virus-induced depletion of uninfected lymphocytes is associated with apoptosis. *Veterinary Immunology and Immunopathology* 104, 33–44.

Schultz, R.D., Thiel, B., Mukhtar, E., Sharp, P. and Larson, L.J. (2010) Age and long-term protective immunity in dogs and cats. *Journal of Comparative Pathology* 142 (Suppl 1), S102–108.

Seehusen, F., Orlando, E.A., Wewetzer, K. and Baumgärtner, W. (2007) Vimentin-positive astrocytes in canine distemper: a target for canine distemper virus especially in chronic demyelinating lesions? *Acta Neuropathologica* 114, 597–608.

Shen, D.T. and Gorham, J.R. (1978) Contact transmission of distemper virus in ferrets. *Research in Veterinary Sciences* 24, 118–119.

Shen, D.T., Gorham, J.R. and Pedersen, V. (1981) Viruria in dogs infected with canine distemper. *Veterinary Medicine & Small Animal Clinician* 76, 1175–1177.

Singethan, K., Müller, N., Schubert, S., Lüttge, D., Krementsov, D.N., Khurana, S.R., Krohne, G., Schneider-Schaulies, S., Thali, M. and Schneider-Schaulies, J. (2008) CD9 clustering and formation of microvilli zippers between contacting cells regulates virus-induced cell fusion. *Traffic* 9, 924–935.

Stein, V.M., Czub, M., Schreiner, N., Moore, P.F., Vandevelde, M., Zurbriggen, A. and Tipold, A. (2004) Microglial cell activation in demyelinating canine distemper lesions. *Journal of Neuroimmunology* 153, 122–131.

Summers, B.A. and Appel, M.J. (1987) Demyelination in canine distemper encephalomyelitis: an ultrastructural analysis. *Journal of Neurocytology* 16, 871–881.

Summers, B.A. and Appel, M.J. (1994) Aspects of canine distemper virus and measles virus encephalomyelitis. *Neuropathology and Applied Neurobiology* 20, 525–534.

Summers, B.A., Greisen, H.A. and Appel, M.J. (1979) Early events in canine distemper demyelinating encephalomyelitis. *Acta Neuropathologica* 46, 1–10.

Summers, B.A., Greisen, H.A. and Appel, M.J. (1983) Does virus persist in the uvea in multiple sclerosis, as in canine distemper encephalomyelitis? *Lancet* 2, 372–375.

Summers, B.A., Greisen, H.A. and Appel, M.J. (1984) Canine distemper encephalomyelitis: variation with virus strain. *Journal of Comparative Pathology* 94, 65–75.

Svitek, N. and von Messling, V. (2007) Early cytokine mRNA expression profiles predict Morbillivirus disease outcome in ferrets. *Virology* 362, 404–410.

Tatsuo, H., Ono, N. and Yanagi, Y. (2001) Morbilliviruses use signaling lymphocyte activation molecules (CD150) as cellular receptors. *Journal of Virology* 75, 5842–5850.

Tipold, A. (1995) Diagnosis of inflammatory and infectious diseases of the central nervous system in dogs: a retrospective study. *Journal of Veterinary Internal Medicine* 9, 304–314.

Tipold, A., Moore, P., Zurbriggen, A., Burgener, I., Barben, G. and Vandevelde, M. (1999) Early T cell response in the central nervous system in canine distemper virus infection. *Acta Neuropathologica* 97, 45–56.

Tsai, S.C., Summers, B.A. and Appel, M.J. (1982) Interferon in cerebrospinal fluid. A marker for viral persistence of canine distemper encephalomyelitis. *Archives of Virology* 72, 257–265.

Vandevelde, M., Kristensen, F., Kristensen, B., Steck, A.J. and Kihm, U. (1982a) Immunological and pathological findings in demyelinating encephalitis associated with canine distemper virus infection. *Acta Neuropathologica* 56, 1–8.

Vandevelde, M., Higgins, R.J., Kristensen, B., Kristensen, F., Steck, A.J. and Kihm, U. (1982b) Demyelination in experimental canine distemper virus infection: immunological, pathologic, and immunohistological studies. *Acta Neuropathologica* 56, 285–293.

Vandevelde, M., Zurbriggen, A., Higgins, R.J. and Palmer, D. (1985) Spread and distribution of viral antigen in nervous canine distemper. *Acta Neuropathologica* 67, 211–218.

Vandevelde, M., Zurbriggen, A., Steck, A. and Bichsel, P. (1986) Studies on the intrathecal humoral immune response in canine distemper encephalitis. *Journal of Neuroimmunology* 11, 41–51.

Visser, I.K., Kumarev, V.P., Orvell, C., de Vries, P., Broeders, H.W., van de Bildt, M.W., Groen, J., Treppema, J.S., Burger, M.C. and UytdeHaag F.G. (1990) Comparison of two morbilliviruses isolated from seals during outbreaks of distemper in north west Europe and Siberia. *Archives of Virology* 111, 149–164.

von Messling, V., Springfeld, C., Devaux, P. and Cattaneo, R. (2003) A ferret model of canine distemper virus virulence and immunosuppression. *Journal of Virology* 77, 12,579–12,591.

von Messling, V., Milosevic, D. and Cattaneo, R. (2004) Tropism illuminated: lymphocyte-based pathways blazed by lethal morbillivirus through the host immune system. *Proceedings of the National Academy of Sciences USA* 101, 14,216–14,221.

Westover, K.M. and Hughes, A.L. (2001) Molecular evolution of viral fusion and matrix protein genes and phylogenetic relationships among the Paramyxoviridae. *Molecular Phylogenetic Evolution* 21, 128–134.

Winters, K.A., Mathes, L.E., Krakowka, S. and Olsen, R.G. (1983) Immunoglobulin class response to canine distemper virus in gnotobiotic dogs. *Veterinary Immunology and Immunopathology* 5, 209–215.

Wünschmann, A., Alldinger, S., Kremmer, E. and Baumgärtner, W. (1999) Identification of CD4+ and CD8+ T cell subsets and B cells in the brain of dogs with spontaneous acute, subacute-, and chronic-demyelinating distemper encephalitis. *Veterinary Immunology and Immunopathology* 67, 101–116.

Wünschmann, A., Kremmer, E. and Baumgärtner, W. (2000) Phenotypical characterization of T and B cell areas in lymphoid tissues of dogs with spontaneous distemper. *Veterinary Immunology and Immunopathology* 73, 83–98.

Wyss-Fluehmann, G., Zurbriggen, A., Vandevelde, M. and Plattet, P. (2010) Canine distemper virus persistence in demyelinating encephalitis by swift intracellular cell-to-cell spread in astrocytes is controlled by the viral attachment protein. *Acta Neuropathologica* 119, 617–630.

Yoshikawa, Y., Ochikubo, F., Matsubara, Y., Tsuruoka, H., Ishii, M., Shirota, L., Nomura, Y., Sugiyama, M. and Yamanouchi, K. (1989) Natural infection with canine distemper virus in a Japanese monkey (*Macaca fuscata*). *Veterinary Microbiology* 20, 193–205.

Zhao, J.J., Zan, X.J., Chai, X.L., Martella, V., Luo, G.L., Zhang, H.L., Gao, H., Liu, Y.X., Bai, X., Zhang, L., Chan, T., Xu, L., Zhao, C.F., Wang, F.X., Shao, X.Q., Wu, W. and Cheng, S.P. (2010) Phylogenetic analysis of the haemagglutinin gene of canine distemper virus strains detected from breeding foxes, raccoon dogs and minks in China. *Veterinary Microbiology* 140, 34–42.

Zurbriggen, A. and Vandevelde, M. (1983) Canine distemper virus-induced glial cell changes in vitro. *Acta Neuropathologica* 62, 51–58.

Zurbriggen, A., Vandevelde, M. and Dumas, M. (1986) Secondary degeneration of oligodendrocytes in canine distemper virus infection in vitro. *Laboratory Investigations* 54, 424–431.

Zurbriggen, A., Yamawaki, M. and Vandevelde, M. (1993) Restricted canine distemper virus infection of oligodendrocytes. *Laboratory Investigations* 68, 277–284.

5 Peste des Petits Ruminants Virus

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5.1 Introduction

Peste des petits ruminants (PPR) is a highly contagious, fatal and economically important disease of both domestic and wild small ruminants, and camels. Owing to high morbidity (100%) and mortality (90%), PPR was included in the OIE (Office International des Epizooties) list of notifiable terrestrial animal diseases. The disease is currently spreading rapidly in most countries of the sub-Saharan and North Africa, the Middle East and Indian sub-continent and as far as into Tibet, China.

During the rinderpest virus (RPV) eradication campaign, there have been significant improvements in understanding the biology of viruses; however, the primary focus has remained developing and improving efficient vaccines. The present chapter aims to provide an overview of all known features of the PPRV genome, structure and biology. The structural and non-structural proteins are described comprehensively. Additionally, available diagnostic tests and potent PPRV vaccines are discussed and finally current challenges and future possibilities for disease eradication are highlighted.

5.2 PPRV Identification and Historical Perspective

Officially, PPR was first described in the Republic of Côte d'Ivoire in West Africa in 1942 (Gargadennec and Lalanne, 1942), however, there are indications that the disease existed much earlier. Since PPR and RP are clinically related diseases and the viruses are antigenically similar, it is believed that PPR remained undiagnosed due to the high prevalence of RP and the inability of the available diagnostic tests to differentiate PPR from RP (Baron *et al.*, 2011). Furthermore, it is likely that, owing to cross-neutralization between PPRV and RPV, small ruminants infected with RPV would have developed protective antibodies suppressing the clinical outcome of PPRV infection (Taylor, 1979). Nevertheless, the disease gained attention when a severe rinderpest-like disease was observed in sheep and goats, which was unable to transmit to the cattle reared in the same herd or in the close vicinity. Initially, different names such as 'kata', 'pseudo rinderpest', 'syndrome of stomatitis-pneumoenteritis' and 'ovine rinderpest' were used to describe the disease. Later, a French name, 'peste des

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petits ruminants', was suggested because of its clinical, pathological and immunological similarities with RPV. At the time of first PPRV recognition, it was considered a variant of RPV. However, Gibbs *et al.* (1979) revealed that PPRV is biologically and physico-chemically distinct and is therefore a new member in the genus *Morbillivirus*, along with RPV, canine and phocine distemper viruses (CDV and PDV), measles virus (MV) and morbilliviruses of porpoises, dolphins and cetaceans (PMV, DMV and CMV).

5.3 Geographical Distribution

After first identification, PPRV spread to sub-Saharan Africa, the Middle East, Turkey and the Indian subcontinent. During the last decade, the disease has been reported for the first time in China, Kenya, Uganda, Tanzania, Morocco and Tunisia (Banyard *et al.*, 2010; Munir *et al.*, 2013). This demonstrates that the virus is highly infectious, and is of emerging transboundary nature. Initially, PPRV was characterized and phylogenetically analysed based on the fusion gene (F), which classified all the strains of PPRV into four distinct lineages (Shaila *et al.*, 1996; Dhar *et al.*, 2002). Later, it appeared that phylogenetic analysis based on the nucleoprotein gene (N) presented a better molecular epidemiological pattern (Kwiatek *et al.*, 2007) and is currently preferred over F gene-based phylogenetic analysis. However, all the PPRV strains remained in the same group regardless of what gene was used as basis for classification, except that the F gene-based lineage I (i.e. Nig/75) became lineage II on the N gene-based tree. Recently, Balamurugan *et al.* (2010) suggested that the use of the haemagglutinin-neuraminidase (HN) gene, in addition to the F and N genes, could give better resolution and permit tracing of virus transmission within outbreaks. Nevertheless, it is still unclear whether differences between lineages merely reflect geographical speciation or if they are also correlated with variability in pathogenicity between isolates (Banyard *et al.*, 2010).

PPRV belonging to lineages I and II have exclusively been isolated from the countries in West Africa, where PPRV once originated. Lineage III is restricted to the Middle East and East Africa. Though lineage IV was strictly considered an Asian lineage, it is now overwhelming the other lineages in African countries, while still being predominant in Asia (Kwiatek *et al.*, 2011; Munir *et al.*, 2013) (Fig. 5.1). Most recent reports of PPRV in previously PPRV-free countries belong to lineage IV, which suggests that lineage IV is a novel group of PPRV and may replace the other lineages in the near future. It is also likely that only lineage IV is currently causing outbreaks. Moreover, it is crucial to note that countries once exclusively carrying a single lineage are now simultaneously reporting the presence of several lineages, i.e. Sudan and Uganda (Kwiatek *et al.*, 2011; Luka *et al.*, 2012). In the majority of cases, the newly introduced lineage is lineage IV (Kwiatek *et al.*, 2011; Luka *et al.*, 2012; Cosseddu *et al.*, 2013).

5.4 Economic Impact of PPR Disease

PPR is generally considered a major constraint for small ruminant production; however, the economic impact of the disease has not been fully evaluated (Ezeokoli *et al.*, 1986; Rossiter and Taylor, 1994; Nanda *et al.*, 1996). The economic importance of PPR is primarily due to its highly contagious nature, with a case fatality rate as high as 100%. This is of particular concern for the economics of small rural farms, where sheep and goats are reared as the sole source of income. Moreover, PPR is most prevalent in countries that rely heavily on subsistence farming of small ruminants for trade and food supply.

The disease consequences can be prevented by the use of highly efficacious vaccine. It has been calculated that an investment of US$2 million can bring a return of US$24 million. This estimation has been made on 1 million animals (Stem, 1993). These facts lead to the perception that PPR is one of the top ten diseases in sheep and goats that are having a high impact on the poor rural small ruminant farmers (Perry *et al.*, 2002). Collectively, it was estimated that PPR causes a loss of US$1.5 million annually in

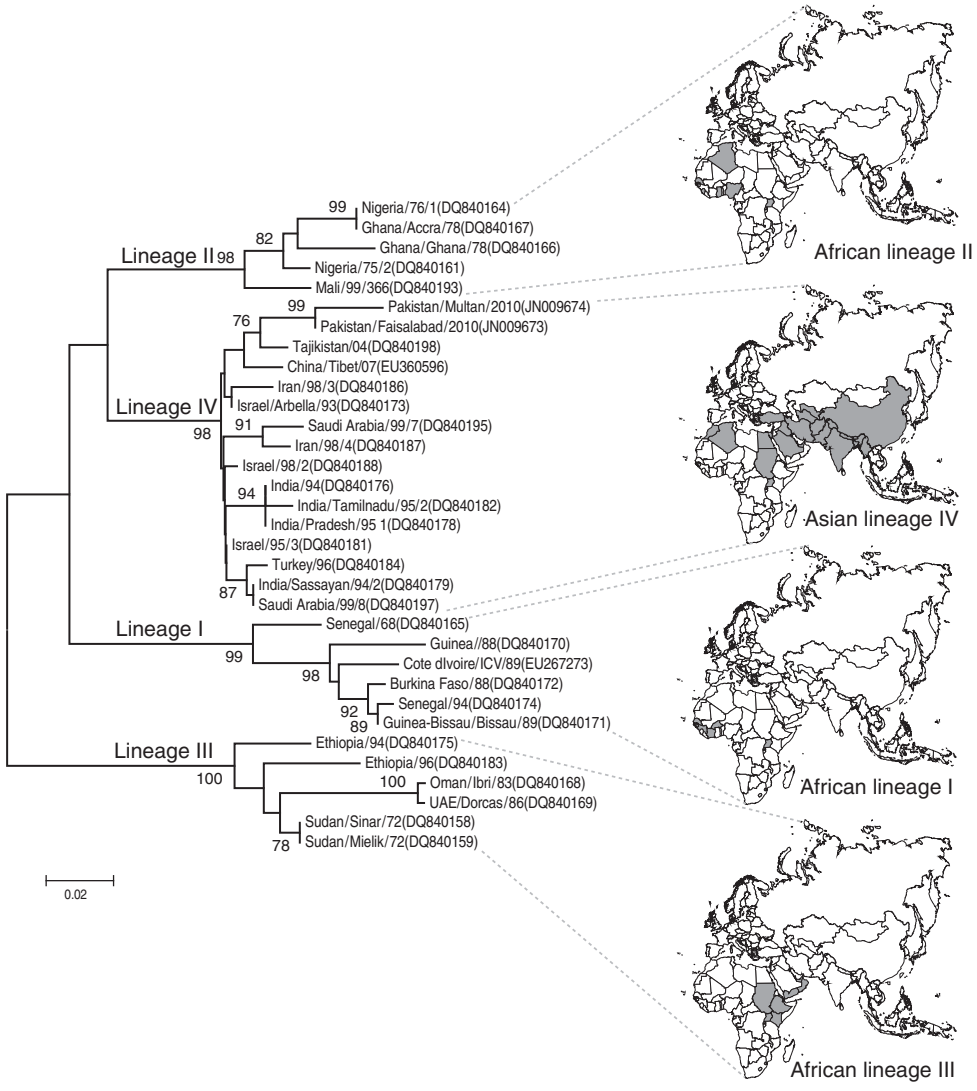

Fig. 5.1. Phylogenetic analysis of PPR isolates based on the N gene. The distribution of different lineages is detailed by shading the maps for each lineage.

Nigeria (Hamdy *et al.*, 1976), US$39 million in India (Bandyopadhyay, 2002) and at least US$1.5 million in Iran (Bazarghani *et al.*, 2006) and US$15 million in Kenya (Thombare and Sinha, 2009). Besides these figures, the worldwide economic impact of PPR largely remains elusive, and a well-planned cost-benefit analysis of PPR versus policy responses that includes both the direct and indirect impacts associated with PPR is required.

5.5 Virion Morphology, Structural and Accessory Proteins

5.5.1 PPR virions

Like other paramyxoviruses, PPR virions are enveloped, pleomorphic particles (Fig. 5.2A) and are comprised of single-stranded RNA genome with negative polarity. The length of the entire genome of PPRV is 15,948

nucleotides, which is the second longest among all morbilliviruses after a recently characterized feline morbillivirus (Bailey *et al.*, 2005; Woo *et al.*, 2012). The diameter of PPR virions ranges from 400 to 500 nm. The phosphoprotein (P) acts as a co-factor of large protein (L), which is the viral RNA dependent RNA polymerase (RdRp). There are three proteins associated with the host cell membrane-derived viral envelope. The matrix (M) protein acts as a link, which associates with the nucleocapsid and the two external viral proteins, the fusion (F) protein and the HN protein. The thickness of the PPRV envelope varies from 8 to 15 nm and the length of the surface glycoproteins ranges from 8.5 to 14.5 nm (Durojaiye *et al.*, 1985).

5.5.2 Viral ribonucleoprotein

The N protein surrounds the genomic RNA along with two other viral proteins, the L protein and the P protein to form the ribonucleoprotein (RNP). This RNP core encloses the entire genome of PPRV and protects from endonuclease digestion. The RNP strands appear as a herring bone with a thickness of ~14–23 nm (Fig. 5.2B) (Durojaiye *et al.*, 1985). Each molecule of N protein is associated with the six nucleotides of the genome, which explains the requirement of 'the rule of six' for paramyxoviruses including PPRV (Lamb and Kolakofsky, 2001). Contrary to this strongly accepted belief, it was revealed that PPRV obey the rule of six but carry a degree of flexibility. By a still unknown mechanism, transcription and replication in PPRV minigenome can accommodate some deviation in genome length, such as +1, +2 and –1 nucleotides (Bailey *et al.*, 2007). Given the fact that the PPRV genome contains 15,948 nucleotides (multiple of six bases), 2650 copies of N proteins are required to completely wrap up the genome. Electron microscopic analysis of nucleocapsid-confirmation in other morbilliviruses (Bhella *et al.*, 2004) indicates that approximately 13 copies of the N protein constitute a single helix, and therefore a genome would involve around 200 turns of the nucleocapsid helix. As is indicated in Fig. 5.2B, the individual cell may contain several copies of the encapsidated RNA PPR virus.

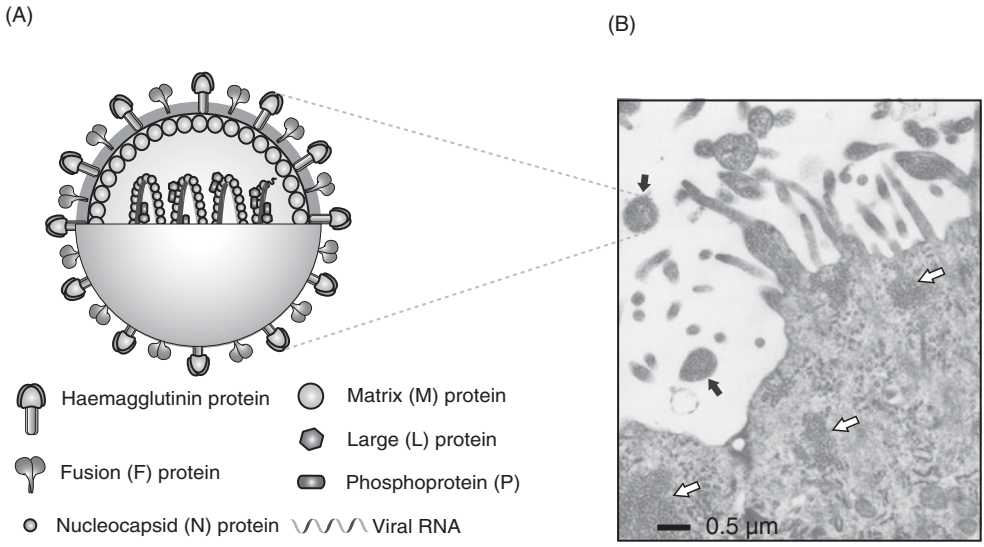

Fig. 5.2. (A) Schematic structure of PPRV, along with a key to different viral proteins. (B) Uranyl acetate and lead citrate staining of the microvilli collected from experimentally infected goats. Herringbone-like cytoplasmic nucleocapsids are labelled with white arrows and the extracellular virions extruding from cytoplasm are labelled with black arrows. (Second part of the figure is reproduced from Bundza *et al.* (1988) with permission.)

5.5.3 Genome organization, replication and transcription

The PPRV genome carries six transcriptional units; each encodes for a contiguous and non-overlapping protein except the P gene, which also expresses C and V nonstructural proteins by an alternative open reading frame and RNA editing, respectively (Mahapatra *et al.*, 2003). All the genes in PPRV are arranged in an order of 3′-N-P/C/V-M-F-HN-L-5′ (Bailey *et al.*, 2005) (Fig. 5.3). An intergenic region of variable lengths separates one gene from the other (Barrett *et al.*, 2006). Notably, owing to variable lengths of the intergenic region between the M and F genes (without having an effect on the protein lengths), the genome varies among morbilliviruses. So far, no obvious role for this variable and high GC content intergenic region has been observed in the replication of the morbilliviruses. The sequence between two consecutive genes is AAAACTTAGGA and is highly conserved throughout the morbilliviruses, including PPRV, indicating that this stretch of sequence is important for viral replications. Data from other paramyxoviruses demonstrate that nucleotides before CTT (underlined in the intergenic sequence) indicate the end of one gene (GE) and are essential polyadenylation sites, whereas the sequence after CTT is the start of next gene (GS).

It has been demonstrated that the 3′ and 5′ untranslated regions (UTRs) at both ends of the paramyxovirus genome, known as genome promoter (GP) and anti-genome promoter (AGP) respectively, are crucial for viral transcription and replication (Lamb and Kolakofsky, 2001) (Fig. 5.3). In PPRV, the 3′-genome terminus, a seat for the attachment of the RdRp polymerase complex, is a stretch of 107 nucleotides, which includes the 52-nucleotide leader region, and 3′ UTR of the N gene, both separated by a trinucleotide (GAA). This stretch of GP before the N gene's open reading frame (ORF) start codon acts as a promoter for the synthesis of viral RNA (Bailey *et al.*, 2007). The gene start and polyadenylation signal are located 52 nucleotides downstream of the N ORF stop in PPRV, which is highly conserved among the morbilliviruses. Recently, an *in vitro* transcription complex was synthesized for PPRV and it was shown that a RNP complex, collected from infected insect cells, is active in synthesizing RNA (Yunus and Shaila, 2012). As with other paramyxoviruses, the N gene present at the 3′ end of the genome was the highest transcribed gene, whereas the L gene present at

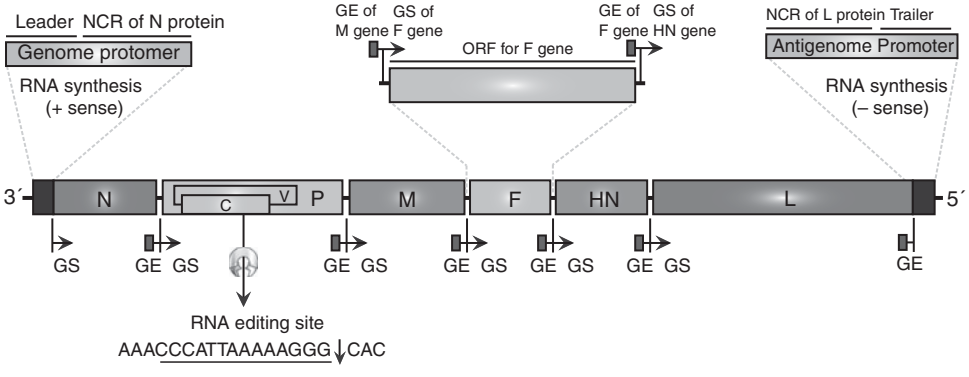

Fig. 5.3. Genome organization of typical PPRV. All the genes are organized in the order of 3′-N-P/C/V-M-F-HN-L-5′, flanked by 3′-genome and 5′-antigenome promoters responsible for the synthesis of + sense RNA and – sense RNA, respectively. Each of the promoters carries sequence for the leader or trailer along with sequence (non-coding region, NCR) from respective genes. Between two genes, there is a stretch of sequence divided into the gene start (GS) of first gene and gene end (GE) of the next gene. It is only P gene that encodes for C and V proteins via alternative open reading frame and RNA editing (insertion of G at the place of arrow after three Gs), respectively.

the end of the genome was the least, owing to attenuation at each gene junction. Quantitative analysis indicated that the level of P mRNA synthesis is 50%, if mRNA for the N gene is taken as 100%, which means that the P gene is transcribed only once out of two attempts of polymerase. Similar analyses show that the synthesis of the L gene mRNA occurs only once out of 75 attempts. Collectively, it was shown that 50% of the total transcripts consist of N mRNA only, and the remaining 50% of all other genes (Yunus and Shaila, 2012). Using this system, it is likely that the post-transcriptional modification activities associated with the L protein of PPRV will also be explored in the near future.

The AGP, which is responsible for the synthesis of genome-sense RNA, is the complement of the 5′ UTR after the L protein stop codon, including the trailer region that becomes the 3′ end of the antigenome. The conserved 3′ and 5′ termini in the entire family reflect the similarity in their promoter activities lying in these regions. A nucleotide stretch of 23–31 at the 3′-terminus of both the GP and the AGP in PPRV is highly conserved and is considered to be an essential domain required for promoter activity. This region is believed to interact with a conserved area comprising a succession of three hexamer motifs (CNNNNN). Although the exact mechanism of domains interaction is unclear, a model has been proposed that predicts that the three hexamer motifs in the second promoter element lie on the same face of the helix, exactly above the first three hexamers at the 3′ terminus (Lamb and Kolakofsky, 2001). It is therefore more likely that these two regions in the GP and AGP interact directly with each other to form a functional promoter unit. A similar assembly is also presented in the promoters of the other paramyxoviruses (Murphy and Parks, 1999). At the junction of the GP and N gene start, a conserved intergenic triplet sequence (CTT) is also considered necessary for transcription (Mioulet *et al.*, 2001). In an effort to construct a minigenome for PPRV, Bailey *et al.* (2007) demonstrated the role of GP and AGP by using chimeric minigenomes of PPRV and RPV. They showed that the use of PPRV-AGP decreased the ability of RPV to rescue the chimeric minigenome, which predicts the difference in closely related viruses. Moreover, it was shown that AGP is a very strong promoter and is responsible for the production of the full-length negative sense genome, whereas the GP is responsible for both transcription of virus mRNAs and transcription of the full-length positive sense virus genome.

5.5.4 Structural proteins

Nucleocapsid (N) protein

Owing to its location at the 3′ end of the genome, the gene that encodes for the N protein is the most transcribed among all genes for both structural and nonstructural proteins of PPRV. The length of the N protein of both PPRV and RPV is 525 amino acids. However, the mobility pattern on SDS-PAGE varies between different strains of PPRV and RPV. It has been observed that the N protein from African isolates (e.g. Nig/75/1) of PPRV moves faster (~55 kDa) than N proteins from the Arabian Peninsula (DORCAS_87) (60 kDa), but migrated slower than the RPV (66 kDa) (Taylor *et al.*, 1990). Therefore, the mobility pattern was considered a biochemical marker for the differentiation of PPRV from RPV and among different strains of PPRV (Lefevre and Diallo, 1990). This difference can be attributed to the post-transcriptional modifications such as glycosylation. Now, due to the availability of the sequences, it is possible to *in Silico* demonstrate that the Arabian isolate DORCAS_87 only has one glycosylation site at the ^{65}NGSK position, whereas the African strain Nig/75/1 has two glycosylation sites at ^{65}NGSK and ^{444}NGSE positions. This difference could contribute to the difference in the mobility of respective strains on SDS-PAGE. Moreover, it is conceivable that N protein is highly susceptible to proteolysis and degradation products may vary between the two viruses and hence differ in molecular weight. However, such predictions require experimental confirmation.

As a common property of paramyxoviruses, the N protein of PPRV is likely to interact with other N proteins (N–N interaction), with the P protein (N–P interaction) and with polymerase units (P–L interaction) to take part in the replication complex. However, only sequences required for the self-assembly of N proteins are mapped for PPRV (Bodjo *et al.*, 2008). It has been demonstrated that two domains, one at the N-terminus (1–120) and one in the central region (146–241), are responsible for the PPRV N–N self-assembly. Additionally, a short fragment in the N protein at amino acid 121–145 is essential for the stability of the resultant nucleocapsid structure.

The N protein plays an essential role in the replication of PPRV (Servan de Almeida *et al.*, 2007). It has been demonstrated that silencing of the N mRNA can block the production of N transcripts and the expression of N protein. Additionally, such shutting down of the N protein indirectly inhibits the production of M protein. Collectively, the shut down of these proteins results in the inhibition of PPRV progeny by 10,000-fold (Keita *et al.*, 2008). A region for efficient siRNA inhibition has now been identified, which is 5′-RRWYYDRNUGGUUYGRG-3′ (where R is A or G, W is A or U, Y is C or U, D is G, A or U and N is any of the four bases). Although this region is found to be common in most of the morbilliviruses, targeting a single region may not prevent the risk of escape mutants. Therefore, in case of therapeutic application of this technique, multiple targets need to be used.

Besides the essential role of the N protein in viral replication and transcription, it regulates host cell protein 72 (hsp72), interferon regulatory factor 3 (IRF3) and cell surface receptors in several morbilliviruses to indirectly promote viral RNA transcription (Zhang *et al.*, 2002; Laine *et al.*, 2003). However, such functions are not described for the N protein of PPRV. The N protein is the most accumulated protein in infected cells and is antigenically most conserved among morbilliviruses (Libeau *et al.*, 1995). Being most abundant, N is a highly immunogenic protein. However, the immune responses generated against the N protein are non-protective due to intra-viral location of the protein. Given its abundance and antigenic stability, the N protein has extensively been targeted for diagnostic assays (Munir *et al.*, 2013). Apart from its diagnostic application, the genetic diversity of the N gene has been the basis for the classification of PPRV into four lineages. This classification better represents the geographical origin than the classification based on the variation of the external glycoprotein, the F protein (Diallo *et al.*, 2007; Kwiatek *et al.*, 2007).

Phosphoprotein (P)

The phosphoprotein of PPRV, as other morbilliviruses, is acidic in nature and undergoes intensive post-translational phosphorylation (hence the acronym phosphoprotein), owing to richness in serine and threonine (Diallo *et al.*, 1987). Due to this, the P protein migrates more slowly (79 kDa) than its predicted molecular weight (60 kDa). The phosphoprotein of PPRV (strain Turkey/00) has a high serine (Ser), threonine (Thr) and tyrosine (Tyr) content (Ser: 38, Thr: 8, Tyr: 5). Approximately 50% of the potential phosphorylation residues in P proteins have high prediction scores (Pred values >0.6); however, potential phosphorylation sites vary between different strains of PPRV.

The length of P proteins varies from 506 to 509 amino acids between different morbillivirus members and the P protein of the PPRV is the longest among all. Despite the essential role of the P protein in viral replication and transcription, it is one of the least conserved proteins, which is demonstrated by the fact that the P proteins from PPRV and RPV share only 51.4% amino acid identity (Mahapatra *et al.*, 2003). Moreover, the region from 21 amino acid to 306 amino acid contains the majority of unconserved residues. Given the fact that the C-terminus of the P protein is involved in the N–P interaction, this terminus is more conserved compared with the N-terminus of the P protein.

In morbilliviruses, the P protein plays crucial roles at multiple levels in both viral

replication and immune regulation. For instance, the N–P interaction is required for key biological processes such as cell cycle control, transcription and translation regulation (Johansson *et al.*, 2003). The motifs required for the interaction of RPV P protein with N protein (N–P interaction) are conserved in the P protein of PPRV. Moreover, the P protein is the vital element of the viral L–polymerase complex, and it is assumed to be a key determinant of cross-species morbillivirus pathogenicity (Yoneda *et al.*, 2004). Despite these crucial roles of the P protein in the replication of morbilliviruses, its function in PPRV replication and pathogenicity remains elusive, which warrants future investigations.

Matrix (M) protein

The ORF for the M protein of PPRV is located at nucleotide position 3438–4442, which is translated to a protein of 335 amino acids with a predicted molecular weight of 37.8 kDa. It is therefore considered one of the smallest proteins among all the structural proteins of morbilliviruses. The protein is highly conserved and a 92.5% and 85.0% similarity and identity have been calculated between PPRV and RPV, respectively. This high degree of conservation may reflect the essential role of the M protein in the formation of progeny viruses and interaction with the surface glycoprotein in the cell membrane. Three ATG repeats (^{956}tctATGATGATGtca^{970}) are identified in the gene for the M protein of PPRV, RPV and MV, while the M gene of CDV, PDV, DMV lacks this domain (Muthuchelvan *et al.*, 2005).

The M protein constitutes the inner coat of the viral envelope and acts as a bridge to connect the surface glycoprotein (F and HN) with that of ribonucleoprotein core (genome, N, P and L) (Fig. 5.2). In an effort to construct a marker vaccine candidate, it was noticed that if the M protein of RPV was replaced with the corresponding protein of PPRV, it did not affect the growth of RPV in cell culture (Mahapatra *et al.*, 2006). Although the mechanism behind compatibility remains to be determined, it at least indicates the high level of M protein conservation among morbilliviruses.

The M protein mediates the viral budding process preferentially at specialized regions of the host membrane. For instance, the budding of MV occurs at the epical microvilli in epithelial cells due to highly concentrated actin filaments, which are required for the cellular transport (Riedl *et al.*, 2002). Electron microscopic images of intestinal epithelial cells from a goat experimentally infected with the Malig-Yemen strain of PPRV indicated that viral particles were released from the microvilli and shed in faeces (Bundza *et al.*, 1988). Moreover, the motif (FMYL) at amino acid position 50–53 required for the localization of the M protein in the cell membrane to facilitate the budding process in Nipah virus, a member of the same family, was found to be identical with that in the M protein of PPRV (Ciancanelli and Basler, 2006). However, it is not known whether these viruses share functional homologies in their M proteins.

Fusion (F) protein

The F protein (59.137 kDA) is one of the highly conserved proteins not only between PPRV and RPV but also among all the morbilliviruses. This conservation probably reflects the cross-protection between PPRV and RPV (Taylor and Abegunde, 1979). In all paramyxoviruses, the F protein is embedded in the viral lipid bilayer envelope and protrudes as spikes on the viral surface (Fig. 5.2). The cleavage of the F protein is a key mechanism of paramyxovirus virulence. The naïve form of the F protein (F0) undergoes post-translational proteolytic cleavage and results in two active subunits, F1 and F2. This mechanism is not well understood for PPRV. However, it has been shown that PPRV carries RRTRR at position 104–108 (Chard *et al.*, 2008), which is recognizable by the *trans*-Golgi associated furin endopeptidase consistent to the cleavage site RRX1X2R (X1 indicates any amino acid, but X2 must be either arginine or lysine) proposed for the morbilliviruses. It has been shown by Rahaman *et al.* (2003) that the membrane-anchoring subunit of F_1

of PPRV contains four well-described conserved motifs: an N-terminus fusion peptide (FP), heptade repeat 1 (HR1), HR2 and a transmembrane (TM) domain. The 3D structure of the HR1–HR2 complex has revealed that the heterodimer between HR2 and HR1 covers the inner core of the HR1 trimer, resulting in a six-helix bundle. The molecular mechanism of PPRV budding is not known, but it is likely based on identical structure of heptade repeats, which have a common fusion mechanism. It has further been shown that on anchoring the FP domain in the membrane, dimerization of the HR domains leads to fusion between the host cell membrane and the viral envelope by bringing them close to each other (Rahaman *et al.*, 2003). A leucine zipper motif, present in the F protein of all the morbilliviruses, is responsible for facilitating the oligomerization and fusion function of the F protein through an unknown mechanism (Plemper *et al.*, 2001). In PPRV, this motif is located at position 459–480 and is conserved among all PPRV strains characterized so far.

In all morbilliviruses, the membrane-associated proteins are glycosylated and hence are known as glycoproteins. This post-transcriptional modification is critical for the transport of the protein to the cell surface, and to maintain its fusogenic ability and integrity. All members of the morbillivirus genus contain a conserved NXS/T (X indicates any amino acid) glycosylation site in the F2 subunit of the mature protein (Meyer and Diallo, 1995). In PPRV, the three N-linked glycosylation sites include ^{25}NLS^{27}, ^{57}NIT^{59} and ^{63}NCT^{65}; however, their specific functions still need to be revealed.

Haemagglutinin-neuraminidase (HN) protein

The ORF for the HN protein gene starts from 7326 and ends at 9152 nucleotide (Nigeria 75/1) and results in a 67 kDa HN protein. The HN protein is the least conserved. While both PPRV and RPV have 609 amino acid residues in their respective HN proteins, the proteins share only 50% amino acid identity. This variation probably reflects the viral specificity for cell tropism and therefore determines the host range. Most of the viral neutralizing antibodies are mainly directed against the HN protein. Hence it is under continuous increased immunological pressure (Renukaradhya *et al.*, 2002). The fundamental roles of the HN proteins in progression of viral infection and specific binding to host cell membrane are not defined in PPRV. However, the findings that the H protein is a major determinant of cell tropism in MV and is the main cause of cross-species pathogenesis in lapinized RPV (Yoneda *et al.*, 2002) indicate that H is the vital antigenic determinant of the morbilliviruses. However, it has been determined that the HN protein of PPRV required a homologous F protein for proper functioning in virus replication (Das *et al.*, 2000).

In some paramyxoviruses, surface proteins can cause haemagglutination and can carry neuraminidase activities. Interestingly, among morbilliviruses it is only MV and PPRV that have haemagglutination capabilities (Varsanyi *et al.*, 1984; Seth and Shaila, 2001). In addition to haemagglutination (viral attachment to cell surfaces and agglutination of erythrocytes), PPRV is unique for its neuraminidase activity (cleaves sialic acid residues from the carbohydrate moieties of glycoproteins). Therefore, it is the only member of the morbilliviruses that has HN protein (Seth and Shaila, 2001), which was previously thought to be absent. RPV, which as already mentioned is very closely related to PPRV, has limited neuraminidase activity but cannot act as a haemagglutinating agent for the erythrocytes (Langedijk *et al.*, 1997). Based on these results, it is suggested to use the more descriptive term HN protein instead of the currently used H protein, as has been used for PPRV in this chapter.

Large (L) protein

The L protein of PPRV is 2183 amino acids long and is regarded as the largest protein in PPR virions. However, due to natural attenuation at each gene-junction in all mononegaviruses, the mRNA encoding for the L protein is the least abundant (Flanagan *et al.*, 2000; Yunus and Shaila, 2012).

Notably, the L protein is conserved among morbilliviruses: PPRV has an identity with RPV and CDV of 70.7% and 57.0%, respectively (Bailey *et al.*, 2005). The protein is rich in leucine and isoleucine, which can be as high as 18.4% (Muthuchelvan *et al.*, 2005). The L protein of PPRV carries a length (2183 amino acids) and molecular weight (247.3 kDa) identical to that of RPV, MV and DMV; however, the protein charge +14.5 is different from those of RPV (+22.0) and PDV (+28.0).

In all morbilliviruses, the L protein acts as RNA-dependent RNA polymerase and performs transcription and replication of the viral genomic RNA. Additionally, the L protein is also responsible for capping, methylation and polyadenylation of viral mRNA. All these steps are crucial for efficient replication of the viruses. Although the direct actions of L proteins are not investigated for PPRV, it is possible to make speculations owing to high sequence identity among morbilliviruses. Three motifs in the L protein have been identified, which are directly linked to the functions of this protein. The corresponding sequences at all these sites are found to be identical in the L protein of PPRV (Munir *et al.*, 2013). The L gene start motif (AGGAGCCAAG) in PPRV, in accordance with the motif found in other morbilliviruses [AGG(A/G)NCCA(A/G)G], is responsible for the generation of viral L gene mRNA and signal for the capping. In PPRV, the corresponding motif required for the binding of L protein with the RNA in morbilliviruses is KETGRLFAKMTYKM at amino acid position 540–553. The sequence ILYPEVHLDSPIV at positions 9–21 can act as a binding site for P and L proteins (Horikami *et al.*, 1994). This sequence for P–L interaction is conserved in paramyxoviruses: in PPRV it is totally conserved except the first amino acid, which is valine instead (Chard *et al.*, 2008). Although most of the important functions of L protein are not defined yet, it is expected that with the current establishment of the reconstituted system for PPRV, it will be possible to demonstrate the multifunctional activities of the L protein of PPRV (Yunus and Shaila, 2012).

5.5.5 Accessory proteins

C protein and V protein

It is only the P gene among all the genes of PPRV that encodes for more than one protein, known as C and V proteins, through alternative open reading frame and RNA editing, respectively, only in virus-infected cells (Mahapatra *et al.*, 2003; Barrett *et al.*, 2006). Apart from the role of C protein in viral replication, recently it has been shown that C protein in RPV inhibits interferon beta (IFN-β) production (Boxer *et al.*, 2009). The molecular mechanism of inhibition still needs to be investigated, but it is likely that the C protein blocks the activation of transcription factors which are required to make up the IFN-β enhanceosome. Whereas the C protein is known to be a virulence factor in MV infection (Patterson *et al.*, 2000) and RPV growth (Baron and Barrett, 2000), the biological function of the C protein in PPRV biology is not known and needs to be examined.

The length of the V protein of PPRV is highly variable among morbilliviruses (Table 5.1). The predicted molecular mass and iso-electric point of the V protein of PPRV is 32.28 kDa and 4.68, respectively. By virtue of having the same initial gene frame, the V protein shares the N-terminus to the P protein, but due to RNA editing, the cysteine-rich C-terminus is different (Mahapatra *et al.*, 2003). The V protein, in contrast to the C protein, undergoes phosphorylation and ~60% of the serine residues are revealed to have a high score for phosphorylation as predicted by Netphos 2.0 (Blom *et al.*, 1999). In the majority of the paramyxoviruses, the V protein antagonizes interferon actions. Studies are required to investigate the functions of the V protein of PPRV, and its relation to other morbilliviruses. My preliminary results indicate that both C and V proteins are associated with IFN regulations at ISRE level in an *in vitro* reporter system. However, the molecular mechanisms of inhibition might differ in both proteins (M. Munir, unpublished data).

Table 5.1. Nucleotide (nt) and amino acid (aa) comparison of open reading frames between genes of PPRV (Turkey/00, AJ849636) and other morbilliviruses.

	Virus/strain (Accession no.)								
	Rinderpest/Kabete 'O' (X98291)		Measles virus/9301B (AB012948)		Canine distemper virus/ Onderstepoort (AF305419)		Dolphin morbillivirus/ CeMV (AJ608288)		Post-translational modification (predicted)
Protein/level	nt	aa	nt	aa	nt	aa	nt	aa	–
Nucleocapsid protein	66.2%	72.9%	66.8%	73.5%	62.5%	68.5%	66.2%	72.9%	Glycosylation
Phosphoprotein	62.4%	50.5%	60.4%	45.1%	56.6%	45.3%	61.6%	49.1%	Phosphorylation
C protein	58.8%	41.8%	53.2%	40.3%	53.1%	35.0%	58.5%	37.2%	–
V protein	61.9%	45.1%	59.0%	41.3%	54.2%	40.4%	59.0%	43.5%	Phosphorylation
Matrix protein	72.2%	66.1%	74.0%	68.2%	73.1%	60.3%	69.1%	64.0%	–
Fusion protein	68.0%	73.8%	67.0%	71.7%	50.2%	54.2%	65.9%	73.3%	Glycosylation
Haemagglutinin/ neuraminidase	55.5%	39.4%	53.5%	34.5%	47.2%	28.4%	52.9%	37.3%	Glycosylation
Large protein	68.1%	75.6%	68.1%	75.1%	64.8%	71.1%	67.3%	73.7%	Glycosylation
Complete genome[a]	63.7%	–	63.4%	–	58.5%	–	62.0%	–	–

[a] Full-length comparison among morbilliviruses. Similarity scores were calculated using BioEdit version 7.0.9.0.

5.6 *In vitro* Cultures and Animal Model

RPV was first successfully grown on bovine kidney cells, but Gilbert and Monnier (1962) were also able to isolate PPRV on primary lamb kidney cells. However, later, because of the problematic quality and considerable variations in primary cultures, an African green monkey kidney (Vero) cell line was used for PPRV isolation (Lefevre and Diallo, 1990). To further improve the isolation method and to reduce the problems associated with the Vero cell line (i.e. low virus isolation, unsuccessful attempts and blind passages) (Abu Elzein *et al.*, 1990; Lefevre and Diallo, 1990), monkey CVI cells expressing sheep–goat signaling lymphocyte activation molecule (SLAM) has been investigated. It was shown that the monkey cell line, designated CHS-20, is highly sensitive for isolation of wild-type PPRV from clinical specimens (Adombi *et al.*, 2011). Studies have shown that SLAM can be a co-receptor for PPRV, which was first confirmed using the small interfering RNA (siRNA) technique (Pawar *et al.*, 2008). Under silenced SLAM receptor in B95a cells (a marmoset lymphoblastoid cell line), PPRV replication was observed to be reduced by 12- to 143-fold, while the virus titre ranged from $\log_{10}$ 1.09 to 2.28 (12–190 times). Taken together, expression and distribution of SLAM was directly proportional to that of PPRV cell tropism, indicating that SLAM may act as a receptor for PPRV infectivity. The mRNA level of SLAM was determined to be higher in lymph nodes and was detectable in the digestive system; however, despite the fact that PPRV also replicates in the lungs, colon and rectum, the SLAM receptors were not activated, which partially demonstrates that SLAM is not the major receptor for PPRV infectivity, and that PPRV additionally relies on other receptors for viral pathogenesis (Meng *et al.*, 2011).

Recently, in an experimental study it was shown that alpine goats are highly susceptible to Morocco strains of PPRV (Hammouchi *et al.*, 2012). The results of this and a corresponding study from the same group concluded that alpine goats can be used for both vaccine and pathogenesis studies in order to consistently reproduce PPR clinical signs in experimentally infected animals (El Harrak *et al.*, 2012).

5.7 Determinants of Virulence

There are several factors that contribute significantly in disease pathology and virus dissemination, some contributed by the host while others are physical factors.

5.7.1 Host factors

Although only domestic and wild small ruminants are considered as the main natural host, PPRV can infect other species such as cattle, pigs, buffalo, camels, and as recently reported, also the Asiatic lion (Balamurugan *et al.*, 2012a). There is little information available about susceptibility, occurrence and severity of the disease in wild ungulate species; however, current literature indicates that wild small ruminants may have a crucial role in the epidemiology of PPR (Munir, 2013). In small ruminants, the severity of the disease may vary depending on age, sex, breed and seasons (Amjad *et al.*, 1996; Brindha *et al.*, 2001; Dhar *et al.*, 2002; Munir *et al.*, 2009; Meng *et al.*, 2011). Generally, it is believed that goats show more severe clinical signs than sheep in the same environmental conditions. This is supported by the fact that the level of PPRV antibodies is higher in sheep than goats, which may render sheep resistant to the disease (Munir *et al.*, 2009). Wosu (1994) has also shown that the rate of recovery is lower in goats than in sheep. The information regarding viral preference for sheep over goats has not been investigated, but it is likely that sheep show higher natural resistance to the disease. Notably, PPRV infection can spread between goats without affecting nearby sheep (Animal Health Australia, 2009), but mixed raising of both sheep and goats is considered to be a main risk factor for seropositivity in sheep flocks (Al-Majali

et al., 2008). It is also plausible that owing to the high fertility rate in goats there may be larger flock replacement by goat offspring, which are more susceptible to the disease than adults due to decrease in maternal antibodies after 4 months (Srinivas and Gopal, 1996; Ahmed *et al.*, 2005). Furthermore, it has been demonstrated that age is the main factor for seropositivity in small ruminants (Waret-Szkuta *et al.*, 2008). The case fatality rate is higher in young goats than in adults (Shankar *et al.*, 1998; Atta-ur-Rahman *et al.*, 2004). Since the males are sold earlier and females are kept for longer, the sex-based distribution of antibodies is usually biased. Goat species from West Africa are more susceptible than European goats (Couacy-Hymann *et al.*, 2007). The dwarf varieties of goats are the most susceptible among African breeds. The disease rate (morbidity) increases with environmental stress such as confinement of animals during winter and rainy seasons (Amjad *et al.*, 1996; Brindha *et al.*, 2001; Dhar *et al.*, 2002). However, the effects of environment on the occurrence of PPR are solely based on the nature of animal husbandry conditions and socio-economic status of the farm owner. Although there have been significant contributions in understanding the risk factors, the genetic marker of disease predisposition are not determined.

5.7.2 Non-host factors

PPRV is highly contagious and in most cases the virus is spread from infected to healthy animals via close contact (Abubakar *et al.*, 2012). However, PPRV is commonly shed in all secretion and excretions, such as from the mouth, eye and nose, and in faeces, semen and urine. Shedding starts after approximately 10 days of pyrexia. Since the virus is also secreted in sneezing and coughing, it is likely that transmission may occur through inhalation or contact with inanimate objects. The survival of PPRV in the dam's milk has not been investigated; however, based on its similarity with RPV, it is likely that PPRV is also secreted in milk 1–2 days before signs appear and as late as 45 days after onset of disease. It has been observed that PPRV-infected animals start virus transmission before the onset of clinical signs (Couacy-Hymann *et al.*, 2007). However, Ezeibe *et al.* (2008) studied the shedding of virus during the post-recovery state of the animal, and realized that goats infected with PPRV can shed virus antigens in faeces for 11 weeks after complete recovery. Little is known about the fragility of PPRV in the external environment. Comparison with RPV is likely to be reliable because there are many features in common. Although transmission is not impossible through fomites, it is not common either, because of the short life of the virus in dry environments (above 70°C) and in acidic (>5.6) or basic (<9.6) pH. Moreover, PPRV cannot exist for a long time outside the host because of its short half-life, which is estimated to be 2.2 minutes at 56°C and 3.3 hours at 37°C (Rossiter and Taylor, 1994). No convalescent carrier or chronic form of PPR has been reported.

5.8 Pathophysiology and Clinical Presentation

Primarily PPRV gains entry into the host via the epithelial lining of the oral cavity, respiratory and digestive tract. Based on this, PPR is also called stomatitis pneumoenteritis complex. Due to its high lymphotropic nature, PPRV replicates in the regional lymph node after internalization. The resultant viraemia facilitates virus dissemination to the surrounding susceptible epithelial tissues of the host. Further replication of the virus in these organs leads to establishment of lesions and clinical signs. The severity of these clinical signs depends on the age, breed, body condition and innate immunity of the host and the virulence of the virus. Moreover, concurrent bacterial and parasitic infections can further aggravate the disease.

Based on these factors, the clinical outcome of the disease is divided into peracute, acute, subacute or subclinical (Braide, 1981; Obi *et al.*, 1983; Kulkarni *et al.*, 1996). However, the acute form of the disease is the most common in both sheep and goats.

Peracute disease is commonly observed in kids and lambs soon after depletion of protective passive immunity. Although this form of disease is less clinically characterized, pyrexia may develop and animals may die 4–5 days post-pyrexia.

In the acute form of the disease, a short incubation period (3–4 days) is followed by pyrexia and severe diarrhoea, which ends in emaciation and prostration. Catarrhal discharges around nostrils can lead to severe dyspnoea, sneezing and coughing (Fig. 5.4A,B). Crusting and congestion of conjunctiva at the medial canthus and conjunctival sac may eventually cause complete closure of the eyelids. Rough necrosis

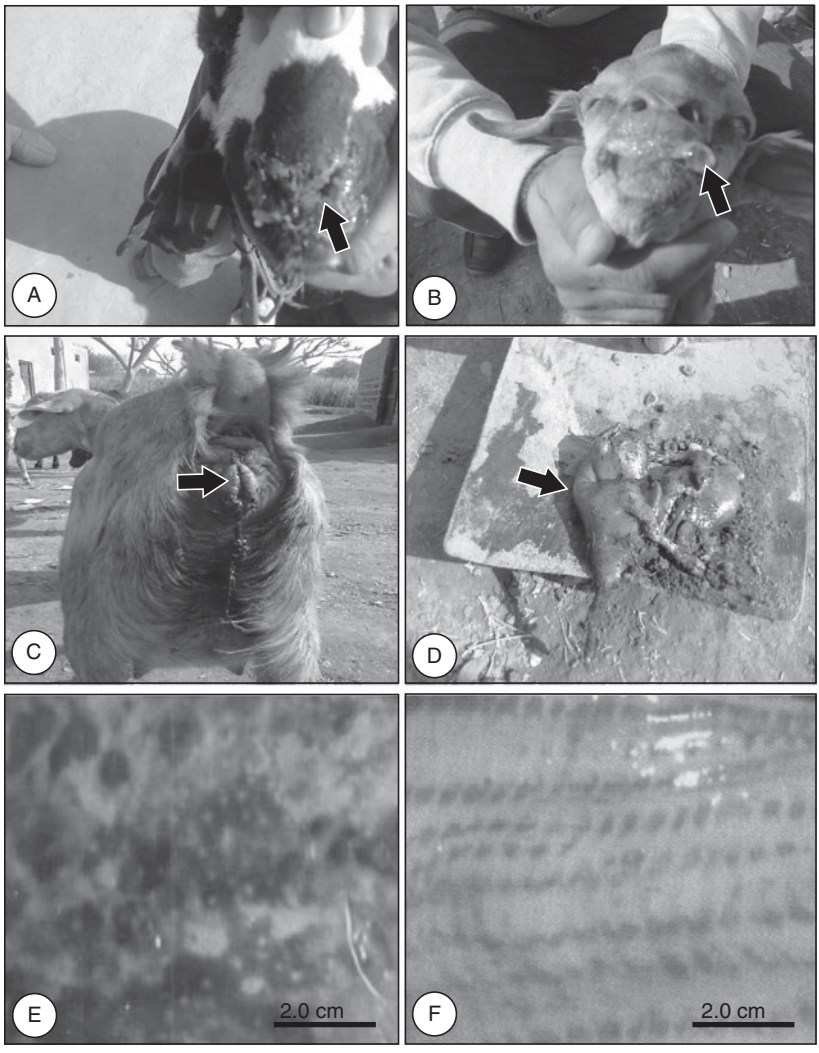

Fig. 5.4. Clinical picture of animals naturally infected with PPRV. (A) The oculo-nasal discharges become catarrhal with disease progression, occlude the nostrils and predispose victim to severe dyspnoea. (B) A serous discharge from the oral cavity and crust on the lips. (C) The pregnant animals may abort. (D) Aborted fetus from the goat shown in Fig. 5.4C. (E) Haemorrhagic erosions on the soft and hard palate. (F) Zebra stripes in the rectum. (A–D were obtained by the author after an outbreak of PPRV in district Multan, Pakistan. The samples were confirmed serologically (cELISA) and genetically (real-time PCR) for PPRV. E and F were modified from Bundza *et al*. (1988) with permission.)

is common on the dental pad, hard palate, inner side of cheek and dorsal part of the tongue, and around the commissures of the mouth. Because of these lesions, animals are reluctant to open their mouth and thus become anorexic. Occasionally, lesions may also develop in the mucous membrane of vulva and the vagina in female animals, which may cause abortion in pregnant animals (Fig. 5.4C,D). The lungs are affected in PPRV-infected animals, causing dyspnoea and productive cough. Severe signs of pneumonia such as noisy respiration with extended head and neck, nostril dilation, protruded tongue and painful cough are indications of poor prognosis. The affected animals then gradually become dehydrated, with sunken eyeballs, and often die 10–12 days post-pyrexia. The case fatality rate ranges from 70 to 80%, while survivors recover after weeks of convalescence.

The subacute form of the disease has a longer incubation period (>6 days) and animals are not as severely affected as in the acute form of the disease. Symptoms similar to contagious ecthyma, such as oral crusts due to mucosal discharges, may appear (Diallo, 2006). After low-grade pyrexia (39–40°C), animals usually recover in 10–14 days, but are immunoprotected enough to prevent re-infection and to protect the offspring for at least the first 3 months.

Subclinical disease is only observed in unusual hosts such as buffalo, as demonstrated by seropositivity against PPRV.

5.9 Gross and Histopathological Lesions

Gross lesions are very typical in the digestive and respiratory systems. In the oral cavity, the major affected sites include the dental pad, hard palate, buccal papillae and the dorsal surface of the tongue. However, ulcerative and necrotic lesions are common on the surface of the oral mucosa, pharynx, upper oesophagus, abomasum and small intestine (Fig. 5.4E). Severe congestions along the longitudinal folds of the caecum, proximal colon and rectum may lead to development of 'zebra striping' (Fig. 5.4F). Oedema and congestion of the lymph nodes may also develop in mesenteric, retropharyngeal and gut-associated lymphoid tissue. In the respiratory system, both anterior and cardiac lobes of the lungs can show severe congestion followed by consolidation and fibrinous or suppurative pneumonia. Hyperaemia, accompanied by frothy exudate, leads to erosions and multifocal ulceration in the mucosa of the nares and trachea. Rarely, focal degenerative lesions are also noticeable in the liver. Bronchitis, tracheitis, atelectasis and interstitial pneumonia may be severe due to secondary bacterial infection.

Some features of PPRV pathology such as atrophy of the villi, reduction of the lymphoid cells in Peyer's patches, dilatation of the cystic crypts of Leiberkuhn with cellular casts and infiltration of the lamina propria with macrophages and lymphocytes have been observed in both naturally and experimentally infected small ruminants. In the liver, hepatomegaly may cause narrowing of the sinusoids. Congestion and hyperplasia of the reticulo-endothelial cells is common in the spleen, whereas coagulative necrosis is common in the kidneys.

In the respiratory tract, histopathological lesions such as multifocal degeneration, ulceration and necrosis are followed by alveolar type II pneumocytes hyperplasia. These lesions lead to syncytial cell formation, which is a prominent feature in the lungs (Aruni *et al.*, 1998; Yener *et al.*, 2004). Multinucleated epithelial giant cells with intranuclear inclusion bodies are also very common among PPRV-infected animals. Notably, this pathology has not been found in the lungs of RPV-infected animals. Some of the most common pathological lesions are shown in Fig. 5.5.

As with several other morbilliviruses, the presence of PPRV in the ependymal cells clearly provides evidence that PPRV has the potential to reach and pass the blood–brain barrier and cause neurovirulence (Kul *et al.*, 2007). The clinical picture of the neurotropic form of PPR in kids and lambs is expected to be severe, especially in the presence of concurrent infections (Kul *et al.*, 2008). Although knowledge about the

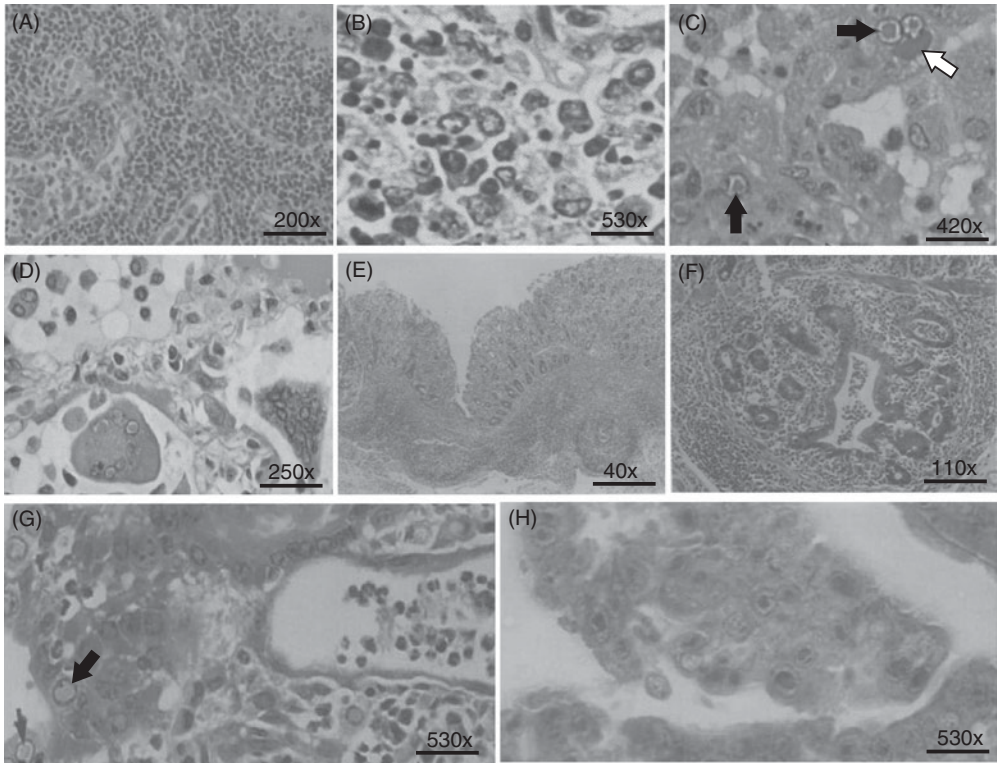

Fig. 5.5. Histopathological lesions of PPRV-infected organs of goats. (A) Macrophages in the germinal centre of the lymph node. (B) Necrosis with pycknosis of the nuclei of the lymph node. (C) Inclusions and oedema in the nucleus (black arrow) and cytoplasm (white arrow) of the lung cells. (D) Formation of oedema, macrophages and alveolar giant cells. (E) Atrophy of the intestinal villi, cellular casts in the crypts, depletion of lymphoid cells in the Peyer's patches. (F) Inflammation of intestinal crypt of PPRV-infected goat. (G) Degeneration of the epithelial cells of the mucosa of the ileum with nuclear inclusions (arrow) and distension of crypts with neutrophils. (H) PPRV antigen in both cytoplasm and nucleus as demonstrated by indirect immunoperoxidase staining. (Modified from Bunza *et al*. (1987) with permission.)

neurotropism of PPRV is in its infancy, it has been shown that the PPRV antigen is detectable in neurons and neuronal processes in the temporal, frontal and olfactory cortices in both hemispheres, and dendrite processes in the telencephalon layer of the hippocampus (Galbraith *et al.*, 1998, 2002).

5.10 Host Responses Against PPRV

5.10.1 Immune responses

Cytokine responses: All eukaryotes have the ability to block the replication of viruses, mediated by several arms of the immune systems such as neutralizing antibodies, the complement system and cytokine production. Animals infected with PPRV show increased expression of type II IFN (IFN-γ), a potent cytokine and crucial player in the direct inhibition of viral replication, in the majority of the epithelial lining the oral cavity, lung and tongue (Atmaca and Kul, 2012). A coordination between IFN-γ and interleukin-4 (IL-4) was observed to be inversely proportional in both vaccinated and infected goats, in which unique biphasic response of IL-4 expression was observed with an up-regulation of IFN-γ (Patel *et al.*, 2012). Besides epithelial cells, an elevated level of IFN-γ can also be observed in intravascular monocytes, syncytial cells, mononuclear

cells and the submucosa of the salivary glands. These results indicate that PPRV has high dissemination capacity and broad tissue tropism. The lungs, interstitial lymphocytes, syncytial cells and alveolar macrophages of PPRV-infected animals also show high expression of TNF-α (Atmaca and Kul, 2012). Due to affinity of the PPRV for the epithelial cells, it is likely that TNF-α plays an active role in stimulation of the cell-mediated immune responses, which warrants further investigations (Opal and DePalo, 2000). It is also likely that an elevated level of inducible nitric oxide synthetase (iNOS) during PPRV infection, in association with TNF-α, may be responsible for the induction of inflammation. Such interpretations have been made for MV in children (Moussallem *et al.*, 2007), whereas infection of ferrets with canine distemper virus showed no induction of cytokine expression in peripheral blood leukocytes (Svitek and von Messling, 2007). The level of IL-4 and IL-10 in PPRV-infected animals can be comparatively high (statistically non-significant) in bronchi, bronchial and interalveolar septum. IL-4 cytokine is responsible for the inhibition of IFN-γ-induced monocytes, whereas IL-10 is primarily involved in the suppression of the production of TNF-α and IL-1. This means that high levels of produced TNF-α and IFN-γ were not significantly affected by the individual or combined effect of IL4 and IL-10. Collectively, the results indicated that TH-2/type-2 cytokines are a critical determinant in deciding the survival of the PPRV-infected animal.

Passive immunity: Passive immunity, the transfer of readymade antibodies from dam to offspring, has been evaluated in naturally infected or vaccinated dams. The newborn kids and lambs acquire these antibodies in colostrum, which are enough to protect them against PPRV for 3–5 months. Different techniques were used to estimate the level of antibodies sufficient to neutralize PPRV and so the times suggested for when kids and lambs should be first vaccinated may vary. Libeau *et al.* (1992) have detected sufficient maternal antibodies until 4 months of age using virus neutralization test but only until the third month using competitive ELISA. Awa *et al.* (2000) demonstrated that protective maternal immunity against PPRV lasts until age of 3.5 to 4.5 months. However, later studies indicated that vaccination of newborns should be started at the age of 3 months in both kids and lambs in PPRV-endemic areas (Bodjo *et al.*, 2006). Although, further support is required, first vaccine at the age of 4 months is accepted in most countries (Balamurugan *et al.*, 2012b).

Cellular and humoral immunity: The nature of the cellular and humoral immunity in recovery of PPRV is not clear yet; however, it was experimentally proven that infection by and vaccination against PPRV generates effective cellular and humoral immunity. Results showed that goats developed both humoral and cell-mediated immune responses against the HN protein of PPRV, and the generated antibodies could neutralize both PPRV and RPV *in vitro* (Sinnathamby *et al.*, 2001). On the other hands, the antibodies exclusively against the F protein of PPRV or RPV are virus neutralizing and are protective against clinical disease (Romero *et al.*, 1994; Berhe *et al.*, 2003).

The mapping of T- or B-cells epitopes (the shortest immunodominant sequence that maintains stimulatory capacity for T- or B-cells) on the viral protein is crucial in designing efficient recombinant vaccines. Studies in both mice and in small ruminants identified a conserved T-cell epitope in the N-terminus (amino acids 123–137) and C-terminus (amino acids 242–609) of HN protein, which is highly conserved between PPRV and RPV (Mitra-Kaushik *et al.*, 2001; Sinnathamby *et al.*, 2001). Using a set of monoclonal antibodies, at least four and two B-cell epitopes were mapped on the N and HN protein of PPRV, respectively (Renukaradhya *et al.*, 2002; Choi *et al.*, 2005). Mitra-Kaushik *et al.* (2001) have demonstrated that the N protein of both PPRV and RPV induces class I restricted, antigenic-specific, cross-reactive strong CD8+ T-cell responses and induces the proliferation of splenic lymphocytes. It was further shown that HN protein of PPRV induces bovine leukocyte antigen (BoLA)

class II restricted helper T-cell responses and BoLA class I restricted cytotoxic T-cell (CTL) responses. The epitope was mapped at amino acid position 408–416 (Sinnathamby *et al.*, 2004). Although the immune responses against the F protein of PPRV remain largely unknown, it is possible to conclude that most of the protective immunity (cellular or humoral) is directed against the surface glycoproteins of PPRV.

5.10.2 Immune suppression

All morbilliviruses cause immunosuppression and therefore predispose the host to secondary bacterial infection, which contributes significantly to the high mortalities associated with morbilliviruses (Beckford *et al.*, 1985). The mechanism of immune suppression in PPRV-infected animals is poorly defined. However, basic studies have shown that a virulent PPRV strain Izatnagar/94 can cause severe leukopenia, lymphopenia and reduction in antibody response (Rajak *et al.*, 2005). Such immunosuppression is evident in the acute phase (4–10 days) of the infection, which coincides with the prominence of clinical disease. The lymphotropic nature of PPRV renders lymphopenia as an important indicator of immune suppression (Raghavendra *et al.*, 1997; Kumar *et al.*, 2001; Rajak *et al.*, 2005). This tropism is linked to the presence of a protein receptor on the cell surface, the SLAM also known as CD150, which is used preferentially by wild-type PPRV to bind to the host (Adombi *et al.*, 2011). It is interesting to note that the vaccine strain of PPRV (Nigeria/75/1) showed profound inhibition of freshly isolated, mitogen-stimulated bovine and caprine peripheral blood lymphocytes (PBL). The level of PBL inhibition was found to be more profound in PPRV (50%) than in a vaccine strain of RPV (30%) in caprine PBL, especially at the high multiplicity of infection (MOI) value of 5 (Heaney *et al.*, 2002). This suppression is of special interest because Nigeria/75/1 is currently being extensively used as live attenuated vaccines. The effect of such immunosuppression on viral secretion and shedding may be of interest for future investigations.

Immune-compromised animals are prone to secondary bacterial and other concurrent infections. In order to investigate the pathology and dissemination of PPRV in immune-compromised animals, Jagtap *et al.* (2012) infected steroid-induced immune-compromised animals with PPRV. These animals not only show an aggravated form of the disease, but interestingly the virus also managed to infect atypical organs such as the liver, kidney and heart, as well as infecting the typical organs. The immunocompromised animals showed viraemia for a short time. However, the rate and extent of disease severity and mortality rate were significantly higher than in non-compromised animals.

In investigations made so far (Rajak *et al.*, 2005; Jagtap *et al.*, 2012), specific antibodies against PPRV were not detected and experiments were only limited to the first 10 days of infections. It is therefore tempting to postulate that PPRV interferes in the induction of humoral immune response. Moreover, host immune responses in the latter stages (>10 days) will be interesting to monitor because mortality usually occurs before seroconversion in the infected animals. Taken together, early detection of PPRV antigens in immunocompromised animals explains the role of these animals in the rapid spread of the disease from sick to healthy susceptible animals in the case of an outbreak. Therefore, the immunosuppressed animals may play a significant role in disease transmission, and can display a severe form of the disease.

The exact mechanism behind PPRV's ability to suppress immunity is not clearly understood. However, based on other antigenic and immunological similarities between morbilliviruses, it is likely that immune-suppression is a multigenic trait. The non-structural proteins of many of paramyxoviruses are associated with immune regulations. For instance, the V protein of MV impairs the production of IFN-α/β, which is mainly mediated by the nuclear factor kappaB (NF-κB) pathway (Caignard *et al.*, 2009). C protein of RPV, in contrast to MV, has been shown to block

the induction of type I IFN (Boxer *et al.*, 2009). In PPRV, the roles of these non-structural proteins (C and V) are not ruled out in antagonizing immune responses and their contribution to viral pathogenesis. The V protein of PPRV has a very similar amino acid identity to the V protein of MV, and therefore it is likely that the IFN inhibitory character of PPRV lies in the V protein. Our preliminary results indicate that both termini of the V protein are involved in the inhibition of IFN-α/β and NF-κB signaling (Munir *et al.*, unpublished data). However, these findings need to be confirmed in both *in vivo* and *in vitro* systems.

5.10.3 Apoptosis

Several viruses cause either inhibition or induction of apoptosis as means of successful replication. Inhibition of apoptosis prevents premature death of the host cells, which results in viral persistence and increases the virus progeny from infected cells. On the other hand, induction of apoptosis helps to release the progeny viruses and the dissemination to neighbouring cells to spread infections. Additionally, induction of apoptosis helps to establish cytotoxicity that facilitates viral pathogenesis (Roulston *et al.*, 1999). PPRV, like other members of the genus, has been shown to induce apoptosis. Infection of goat peripheral blood mononuclear cells (PBMC) with PPRV resulted in peripheral condensation of chromatin, blebbing of plasma membrane, fragmentation of the nucleus and cell leading to formation of apoptotic bodies (Fig. 5.6A,B,C) (Mondal *et al.*, 2001). All these deformities are typical for the induction of apoptosis. Although the mechanism associated with PPRV-induced apoptosis is not well understood, it has been noticed that induction of apoptosis was directly proportional to that of virus replication, which indicates that PPRV exploited the programmed cell death in favour of its replication (Fig. 5.6D). The roles of different viral proteins in regulating apoptosis have been investigated for MV, a member of morbilliviruses that shares several features of PPRV. Recently, it has been shown that the nucleoprotein of MV induces apoptosis (Bhaskar *et al.*, 2011). Owing to the high genetic similarity between N proteins of PPRV and MV (Table 5.1), it is plausible that the N protein of PPRV is also responsible for regulating apoptosis. It is also highly likely that induction of apoptosis might be associated with immune suppression, as in the case of MV (Schnorr *et al.*, 1997). Since the molecular mechanisms of immune suppression in PPRV infection have not been investigated so far, such speculations require future research to underpin the mechanisms involved in these pathways.

5.10.4 Haematological responses

Natural infection by PPRV causes severe haemorrhages in the alimentary canal and in the liver. These haemorrhages and severe diarrhoea in kids lead to decrease in erythrocytes and haematocrit values (Sahinduran *et al.*, 2012). Since PPRV replicates in lymphoid organs, it can also cause leukopenia, characterized by monocytopenia, and lymphopenia (Aikhuomobhogbe and Orheruata, 2009; Sahinduran *et al.*, 2012). It has also been demonstrated that PPRV infection in kids causes significant thrombocytopenia (decrease in thrombocytes) and increases the activated partial thromboplastin time (APLTT) and prothrombin time (PT). It has been suggested that decreased production of thrombocytes or platelets (PLT) from bone marrow, increased consumption of PLT, loss of PLT due to peripheral destruction, or a combination of these factors can lead to increase in both APLTT and PT, markers that determine the clotting tendency of blood. However, trauma and disseminated intravascular coagulation in the PPRV-infected liver can also significantly delay APLTT and PT. Infection by PPRV can also cause an increase in globulin (part of the immune system) and decrease in albumin (which regulates the colloidal osmotic pressure of blood) compared with non-infected animals (Yarim *et al.*, 2006).

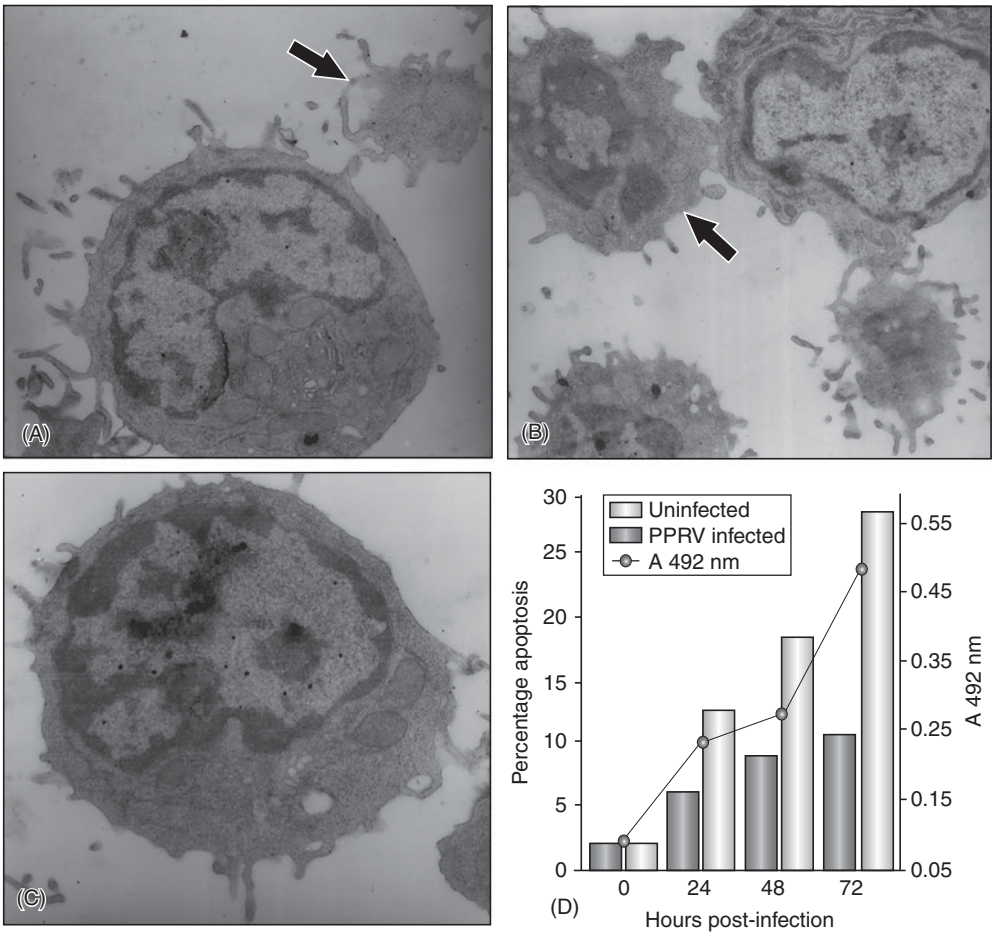

Fig. 5.6. Apoptosis induction by PPRV infection. (A) Goat cells show deformities in PPRV-infected cells, such as margination of chromatin and blebbing of the plasma membrane. (B) The infected cells showing the formation of apoptotic bodies. (C) Non-PPRV infected normal cells without any deformity. (D) The level of apoptosis is correlated with the replication of PPRV. (Modified from Mondal *et al.* (2001) with permission.)

5.10.5 Biochemical responses

PPRV has a high tendency to replicate in the kidney cells and therefore cause increase in blood urea nitrogen and creatinine, both of which are considered markers of renal function (Sahinduran *et al.*, 2012). There are several enzymes, such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase and gamma glutamyl-transferase (GGT), that are considered markers of liver function, all of which are significantly increased in PPRV-infected animals except GGT (Yarim *et al.*, 2006; Sahinduran *et al.*, 2012). Bilirubin is processed first in the spleen (direct bilirubin) and then in the liver (indirect bilirubin). Infection of PPRV can cause significant increase in the elevated levels of both direct and indirect bilirubin, and hence the total bilirubin in the blood serum. However, the cholesterol level (another indicator of devastating diseases) is unaffected in PPRV-infected or non-infected animals. Disturbed levels of sialic acid, a component of cell membrane and receptor for PPRV, indicate the presence of acute phases of the disease. An elevated level of sialic acid was observed in PPRV-infected animals compared with non-infected animals.

Cell-mediated immune responses may also lead to an increase in sialic acid in serum. Regardless of the cause of this induction, Yarim *et al.* (2006) suggested that serum sialic acid can be used as a marker for the diagnosis of PPRV infection in small ruminants.

5.11 Genetics and Reverse Genetics

The production of live virus entirely from cloned DNA (reverse genetics) is a fundamental step in understanding not only the biology of any virus but also to construct marker and DIVA vaccines. The greatest hurdle in the progression of PPRV research has probably been the lack of a reverse genetic system. The reverse genetics for RPV, a virus that is most closely related to PPRV, was established in 1997 using an approach that was similar to that used for rabies virus, another member of family paramyxoviridae (Schnell *et al.*, 1994; Baron and Barrett, 1997). Adapting such strategies for PPRV remained unsuccessful. However, several studies have led to a better understanding of the essential role of PPRV genes. It was shown that RPV has the capacity to accommodate several viral genes of PPRV, including HN, F and M or a combination of these. Additionally, it was shown that a compatible set of HN and F genes from a homologous system is required for satisfactory replication of RPV, which can further be improved with the inclusion of the M gene (Das *et al.*, 2000; Mahapatra *et al.*, 2006; Parida *et al.*, 2007). Using these results, a functional minigenomic replication system for PPRV was generated (Bailey *et al.*, 2007). Now, with the availability of complete genome sequences of several PPRV strains and necessary information for viral replication, a reverse genetic system has been successfully reported (Hu *et al.*, 2012). The major attempt was made to replace T7-RNA-polymerase with RNA-pol-II promoter to initiate transcription. It was thought that due to the presence of sequence elements in the PPRV genome (e.g. cryptic transcription termination signals), the transcription might have terminated prematurely. This system, which is based on the PPRV strain Nig/75/1 (vaccine strain), has the capacity to carry a foreign gene (green fluorescent gene) without having a significant effect on viral replication. This suggests that the infectious clone has the potential to be used for both DIVA and to construct recombinant multivalent vaccines by replacing the GFP ORF with the coding sequence for an immunogenic antigen from another virus, or the creation of tagged viruses for use in fundamental research on the growth and spread of PPRV in its hosts. However, the effect of knockout of a specific gene on both viral replication and its virulence remains to be determined. Additionally, it would be interesting to repeat this reverse genetic strategy for any field strains of PPRV to understand the contribution of individual genes in viral pathogenesis.

5.12 Diagnosis

Earliest possible diagnosis of PPR is crucial in implementing control measures, to contain outbreaks and minimize economic losses. Initially, the majority of PPR outbreaks were diagnosed based on typical clinical signs. However, the signs of PPR are often difficult to distinguish from those caused by a number of other diseases, such as foot-and-mouth disease and bluetongue disease (Munir *et al.*, 2013). This situation becomes even more complicated when these diseases are circulating in areas where PPR is endemic. Thus, it is necessary to confirm the clinical diagnosis through laboratory testing (Munir *et al.*, 2013). Currently, the diagnosis of PPRV is made based on demonstration of antibodies, which is a good indication because an animal infected with PPRV carries antibodies for life, with the development of a sustained antibody response.

5.12.1 Serological detection

Most of the available diagnostic assays have been developed based on the N protein. Owing to the presence at the 3′ end of the genome of PPRV, the N protein produced in quantities

higher than any other structural proteins because attenuation occurs at each intergenic region between two genes (Lefevre *et al.*, 1991; Yunus and Shaila, 2012). The antibodies produced against the N protein don't protect the animals from the disease. Due to abundance of the N protein it remains the most acceptable target for the design of PPRV diagnostic tools (Diallo *et al.*, 1994). Moreover, because the HN protein is the most diverse among all the members of morbilliviruses, RPV and PPRV share only 50% similarity in their HN proteins. The HN protein determines cell tropism; most of the protective host immune response is raised against HN protein. Therefore, serological assays have also been developed targeting HN protein (Munir *et al.*, 2012a, 2013). Commercial ELISAs are available based either on the HN (Saliki *et al.*, 1993; Anderson and McKay, 1994; Singh *et al.*, 2004) or N proteins (Libeau *et al.*, 1995) for specific detection of antibodies against PPRV, in any susceptible host. The sensitivity and specificity of these assays can be as high as 90% and 99%, respectively.

5.12.2 Antigen detection

Immunocapture (Libeau *et al.*, 1994) and sandwich ELISAs (Saliki *et al.*, 1994) are available to efficiently detect antigens in the tissues and secretions of PPRV-infected animals. Both these assays utilize monoclonal antibodies (MAbs) directed against the N protein of PPRV. Both assays are rapid, sensitive and specific with a detection limit of $10^{0.6}$ $TCID_{50}$/well. Since the MAbs used in these assays are raised against the non-overlapping domains of the N protein of PPR and RP viruses, this assay can be used to differentiate PPRV- from RPV-infected animals (Libeau *et al.*, 1994).

5.12.3 Genome detection

To overcome several shortcomings of the serological and antigen detections, such as the requirement of sera in well-preserved format, several PCRs have been developed for PPRV with wide range of sensitivities, specificities and detection limits (Munir *et al.*, 2013). Despite the high sensitivity and specificity of these assays, and their validity to detect both vaccine and field viruses, none of the assays is a formally approved OIE method. For this they need further extensive validation. None of the assays is field applicable since they require thermocycler and electrophoresis apparatus for RT-PCR, and real-time PCR for probe or SYBR-Green-based assays. However, with the development of LAMP assay, on-site detection can be proposed. It is highly plausible to combine the simple procedures for RNA extraction using Whatman FTA card (Munir *et al.*, 2012b, 2012c) and using the RT-LAMP assay for isothermal amplification. This could possibly be applied for field diagnosis of PPRV. Recently, a novel and non-amplification strategy was proposed in which two probes complementary to the target sequences (one conjugated to magnetic microparticles, the second to gold nanoparticles labelled with horseradish peroxidase) were used (Tao *et al.*, 2012). On specific binding to the target, the system allows magnetic separation and substrate detection. It was proposed to be quick (45 minutes), cheap and sensitive (17.6 ng/µl) for PPRV detection. This method holds great potential, especially when it is multiplexed for the detection of several pathogens in the same clinical sample.

5.13 Vaccines Against PPRV

The immunosuppression, caused by PPRV, predisposes the animals to secondary infections. However, through an unknown mechanism, recovery from the infection is usually followed by the establishment of a strong, specific and long-term protective immune response (Cosby *et al.*, 2005). Post-infection or vaccination immunity provided solid foundations for implementation of effective control strategies. The reverse genetic system has recently been established, which is

expected to revolutionize the recombinant vaccines with added value of DIVA. Owing to structural, genetic and antigenic similarities with RPV, significant improvements have been made in the vaccine developments against PPRV. All available PPRV vaccines can be divided into the following categories.

5.13.1 Serum immunization

Inoculation of hyperimmune serum, collected from recovered or infected animals, to susceptible animals at the fever stage could protect them from PPRV for at least 10 days, before the infection reappears (Ihemelandu *et al.*, 1985). However, this short-term passive immunization can be prolonged for to up to 9 months when hyperimmune serum is inoculated along with a virulent strain of PPRV (Adu and Joannis, 1984). This method of protection is now not often practised because of the high cost of obtaining hyperimmune serum, the unavailability of a virulent strain of PPRV and the short shelf life of virulent blood (<10 days).

5.13.2 Heterologous vaccines

After first recognition of PPRV, efforts were made to establish attenuated PPRV for immunization purposes. However, when such efforts failed until 65th cell-culture passages (Gilbert and Monnier, 1962), a RP vaccine was applied in the field to protect animals against PPRV (Bourdin *et al.*, 1970; Bonniwell, 1980). Ultimately, owing to cross-protection between RPV and PPRV, an attenuated Plowright's tissue culture RP vaccine (TCRPV) was used in many countries. This vaccine was considered safe in pregnant goats (Adu and Nawathe, 1981), and the upcoming kids carried passive immunity for at least 3 months. The vaccinated animals are protected against PPRV for at least 3 years (Rossiter, 2004), which is a consequence of strong cross-cellular immune responses. However, because of the intense effort to eradicate RPV to attain the status of RP-free countries, the use of such vaccine was discouraged. The global eradication of RP on 25 May 2011 necessitated the use of only homologous PPRV vaccine in small ruminants.

5.13.3 Homologous vaccines

In an effort to establish a homologous vaccine system for PPRV, Gilbert and Monnier (1962) successfully grew PPRV in primary cell culture, where they observed large syncytia formation as a cytopathic effect (CPE). However, despite early virus isolation, Diallo *et al.* (1989) reported a PPRV that is attenuated in cell culture, and established the bases for a homologous vaccine against PPRV. The PPRV isolated by Taylor and Abegunde (1979) from Nigerian goats that had died from PPRV infection in 1975 (Nig/75) was adapted to Vero cells at 37°C. The isolate was proved to be a powerful substitute for the heterologous vaccines. Currently, in most PPRV-endemic countries, Nig/75 is being used in the production of live attenuated vaccines (Taylor and Abegunde, 1979). The effective dose was calculated to be $10^{0.8}$ TCID50/animal; however, a dose of 10^{3} TCID50/animal also proved to be safe (Martrenchar *et al.*, 1997). Pregnant animals remained safe and were able to pass passive immunity to their offspring, which remained protected for 3–5 months. Later, the vaccine in the field was shown to be protective against wild-type PPRV virus, and immunized animals were also protected against RPV. Beside Nig/75/1, three vaccines of Indian-origin PPRV (Sungri/96, Arasur/87 and Coimbatore/97) have also been assessed as efficient in protecting against field strains of PPRV (Saravanan *et al.*, 2010). Another strain of PPRV, Egypt/87, has been attenuated and has been commercialized by the Veterinary Serum and Vaccine Research Institute, Egypt (Nahed *et al.*, 2004).

Although all the above-mentioned vaccines are highly efficacious, they are susceptible to thermodegradation. For complete immunization, these vaccines need to be

shipped at 2–8°C and should be stored at –20°C, which is difficult in the generally tropical and subtropical countries where PPR is endemic. There have been serious efforts to improve the thermostability of these vaccines (Sen *et al.*, 2010; Munir *et al.*, 2013), but with limited success, which is why the production of recombinant vaccines is still needed.

5.13.4 Recombinant marker vaccines

The surface glycoproteins (F and HN) of PPRV and RPV have been used to cross protect against the viruses. To establish heterologous marker vaccines, it has been shown that a recombinant vaccinia virus, expressing H and F proteins of RPV, is protective against both PPRV and RPV (Jones *et al.*, 1993). Similarly, a recombinant capripox virus containing either the H or F genes of RPV established complete protection against challenged PPRV (Romero *et al.*, 1995).

Again targeting the surface glycoproteins (F protein), a multivalent capripox virus-based vaccine has been reported. A capripox expressing the PPR F protein was protective for both PPRV (Guinea-Bissau/89) and capripox (Yemen isolate) (Berhe *et al.*, 2003). Moreover, a reduced dose for effective immunization was further suggested as an economical way of protecting against both diseases. Animals immunized with a baculovirus expressing HN protein construct produced a high level of neutralizing antibody responses, bovine leukocyte antigen (BoLA) class II restricted helper T cell responses and BoLA class I restricted cytotoxic T cell (CTL) responses (Sinnathamby *et al.*, 2004). Collectively, these responses protected the immunized animals against both PPRV and RPV. Baculoviruses can infect a wide variety of mammalian cells, without being able to replicate, and can therefore be used as an efficient system for delivering recombinant baculovirus-based vaccines to antigen-presenting cells for better immune responses (Ghosh *et al.*, 2002). Intraperitoneal immunization of BALB/c mice with a recombinant *Bombyx mori* nucleopolyhedrovirus (BmNPV), having ability to express antigenic epitopes of the F protein of PPRV and the H protein of RPV, lead to a high level of neutralizing antibodies against respective genes (Rahman *et al.*, 2003). It was further concluded that the use of *B. mori* larvae for large-scale production of recombinant antigens in lieu of the cell-culture system is the most economical means of protection against PPRV. The HN protein of PPRV maintained its immunodominant epitopes in its natural confirmation when expressed in peanut plants (*Arachis hypogea*). The inoculated sheep remained immunogenic and were able to express anti-PPRV-HN protein-specific cell-mediated immune responses without addition of any adjuvant (Khandelwal *et al.*, 2011).

Recently, a replication-competent recombinant canine adenovirus type-2 (CAV-2) expressing the HN gene of PPRV (China/Tibet strain) was generated (Qin *et al.*, 2012). Immunization of goats with this construct provided sterile immunity in term of protective antibodies and PPRV neutralizing antibodies even after primary infection and a profound response was observed with the booster dose. The level of antibodies remained in the goats for at least 7 months and the inoculated virus was not detected in urine or in faeces until as late as 35 days post-vaccination. Although this directly indicates the efficacy of the vaccine, animal sterility and virus shedding for longer period remain to be determined. Taken together, these are successful heterologous marker vaccines, and can be used while there are no efficient live attenuated vaccines specifically designed for PPRV.

5.13.5 Subunit vaccines

Because a PPRV infectious clone was not available until recently, efforts were made to construct subunit vaccines based on the available reverse genetic system for RPV. In the initial studies, to create a RPV in which either the F or HN gene were replaced with the corresponding genes of PPRV, it was revealed that homolog surface glycoproteins

are crucial for viral replication (Das *et al.*, 2000). However, the replacement of both genes allowed the RPV to grow comparatively better. Nevertheless, the construct provided protective immunity in vaccinated animals against challenged wild-type PPRV. To improve the replication efficacy, a triple chimeric RPV expressing the M gene, in addition to F and HN, was constructed (Mahapatra *et al.*, 2006). Interestingly, the chimeric virus grew as high as unmodified PPRV, but comparatively lower than the parental RPV. However, it remained as protective as dual chimeric virus. In the following year, the same group created a chimeric RPV that expressed the N protein derived from PPRV and suggested its use as a marker vaccine (Parida *et al.*, 2007). Recently, Buczkowski *et al.* (2012) have described a novel mechanism of marking morbillivirus vaccines, using RPV as a proof of concept, and they discuss the applicability of this method to the development of marked vaccines for PPRV. Although an efficient DIVA system is still lacking, results described above provide enough information to suggest an efficient system to discriminate infected from vaccinated animals.

5.13.6 Multivalent vaccines

Given the fact that PPRV has been reported concurrently with bluetongue virus (BTV) (Mondal *et al.*, 2009), sheep poxvirus (SPV), goat pox virus (GPV) (Saravanan *et al.*, 2007) and pestivirus (Kul *et al.*, 2008), it is essential to design multivalent (bi- or trivalent) vaccines to support economic vaccination infrastructures in developing countries. Considerable effort has been made to develop multivalent vaccine again targeting surface glycoproteins (F and HN proteins). Recombinant capripox viruses expressing HN protein (Diallo *et al.*, 2002) or F protein (Berhe *et al.*, 2003) were protective for both goat pox and PPR. However, for complete protection, a higher dose of capripox expressing HN protein was required compared to the one expressing F protein. The ability to neutralize PPRV and subsequent inhibition of viral secretion were not determined in any of these vaccines. Recently, a study conducted by Chen *et al.* (2010) showed that recombinant capripoxviruses expressing HN protein (rCPV-PPRVHN) were high inducers of virus-neutralizing antibodies than capripoxviruses expressing F protein (rCPV-PPRVF). The results have provided evidence that PPRV and goatpox viruses don't interfere in each other's immunogenicity, and that these may provide suitable bivalent vaccines in regions where both these diseases are prevalent. However, the duration of immunity conferred by the combined vaccine and field application of these vaccines still remain to be determined.

5.13.7 PPRV replication inhibition by RNA interference (RNAi)

A trend in establishing novel means of PPRV control led to a novel technique derived from molecular genetics. Silencing the expression of N protein by the use of RNAi can lead to reduction in the virus replication *in vitro* by 80% (Servan de Almeida *et al.*, 2007). Although field application and use of such control strategies *in vivo* is questionable, it is expected that investment in novel control strategies will open the way and provide foundations for understanding the biology of PPRV.

5.14 Control and Challenges

After successful eradication of RP, efforts are now being made to control and eradicate PPR, which appeared to be the most appropriate target owing to identical features of the virus, disease mechanisms and epidemiological patterns. Having this aim in mind, there are factors that can favour control and eradication of PPRV. Efficient vaccines are available for immunization and sensitive assays are available to detect the virus in all possible clinical samples. Significant efforts are now being made to improve thermostability of the vaccines and field applicability of the assays. Although

there appear to be four lineages, there is only one serotype for PPRV. The host spectrum of PPRV is relatively narrow with small ruminants as the dominant host, compared with several other infectious diseases. However, this range is now extended to most wild small ruminants and camels. The requirement of close contact for disease transmission, short incubation period (2–6 days) and life-long seropositivity further favour the control and eradication of the disease. With the availability of a reverse genetic system, novel recombinant, multivalent and also DIVA vaccines, the global eradication of PPR is feasible and achievable. On the other hand, there are factors that constrain global eradication. PPR eradication cannot be completely realized without evoking its spearhead role in animal health. The full economic consequences of the disease have not been determined. Vaccines need to be cost-effective and available in developing and PPRV-endemic countries. The overall prevalence of PPR, especially in unusual hosts such as wild small ruminants and camels, and their role in disease epizootiology, need to be investigated before promising any effort for control and eradication of the disease. Recent identification of PPRV in Asiatic lion (*Panthera leo persica*) added another dimension in the host-spectrum of PPRV (Balamurugan *et al*., 2012a). Therefore, monitoring of several wildlife species for presence of the PPRV needs to be considered.

On the other hand, due to the fact that small ruminants have a lower value compared with cattle, it is a matter of relevance when comparing the success of the RP campaign with a future PPR eradication. Moreover, because goats have a shorter lifespan and thus shorter generations, they are feeding the population with naïve individuals at a higher rate than cattle. Generally, women keep goats, whereas men manage cattle. The challenge is therefore not only biological or logistical, but also depends on societal and economic aspects. Nevertheless, a unified framework, as was in place for the RP eradication programme, is currently lacking and needs the urgent attention of international organizations, such as FAO and OIE.

5.15 Conclusions

In conclusion, despite some advances, the mechanism of infection and pathogenesis of PPRV warrants future investigation. The mechanisms of host–pathogen interaction require proper investigations to not only estimate the disease outcome, but also to provide foundations for the future DIVA and marker vaccines. Our current understanding is that PPRV differs to certain degrees from members of the same genus, which highlights the need to investigate the differences. These observations will help to estimate the host-range spectrum of the viruses in unusual hosts. Collectively, the evaluation of its economic impact, improvement and commercialization of diagnostic tests and vaccines, and coordination and integration for planning eradication are key elements to be considered in the global eradication of PPR.

References

Abu Elzein, E.M., Hassanien, M.M., Al-Afaleq, A.I., Abd Elhadi, M.A. and Housawi, F.M. (1990) Isolation of peste des petits ruminants from goats in Saudi Arabia. *Veterinary Record* 127, 309–310.

Abubakar, M., Arshed, M.J., Zahur, A.B., Ali, Q. and Banyard, A.C. (2012) Natural infection with peste des petits ruminants virus: a pre and post vaccinal assessment following an outbreak scenario. *Virus Research* 167, 43–47.

Adombi, C.M., Lelenta, M., Lamien, C.E., Shamaki, D., Koffi, Y.M., Traore, A., Silber, R., Couacy-Hymann, E., Bodjo, S.C., Djaman, J.A., Luckins, A.G. and Diallo, A. (2011) Monkey CV1 cell line expressing the sheep-goat SLAM protein: a highly sensitive cell line for the isolation of peste des petits ruminants virus from pathological specimens. *Journal of Virological Methods* 173, 306–313.

Adu, F.D. and Joannis, T.E. (1984) Serum-virus simultaneous method of immunisation against peste des petits ruminants. *Tropical Animal Health and Production* 16, 119–122.

Adu, F.D. and Nawathe, D.R. (1981) Safety of tissue culture rinderpest vaccine in pregnant goats. *Tropical Animal Health and Production* 13, 166.

Ahmed, K., Jamal, S.M., Ali, Q. and Hussain, M. (2005) An outbreak of peste des petits ruminants in goat flock in Okara, Pakistan. *Pakistan Veterinary Journal* 25, 146–148.

Aikhuomobhogbe, P.U. and Orheruata, A.M. (2009) Haematological and blood biochemical indices of West African dwarf goats vaccinated against Pestes des petit ruminants (PPR). *African Journal of Biotechnology* 5, 743–748.

Al-Majali, A.M., Hussain, N.O., Amarin, N.M. and Majok, A.A. (2008) Seroprevalence of, and risk factors for, peste des petits ruminants in sheep and goats in Northern Jordan. *Preventive Veterinary Medicine* 85, 1–8.

Amjad, H., Qamar Ul, I., Forsyth, M., Barrett, T. and Rossiter, P.B. (1996) Peste des petits ruminants in goats in Pakistan. *Veterinary Reccord* 139, 118–119.

Anderson, J. and McKay, J.A. (1994) The detection of antibodies against peste des petits ruminants virus in cattle, sheep and goats and the possible implications to rinderpest control programmes. *Epidemiology and Infection* 112, 225–231.

Animal Health Australia (2009) *Disease strategy: Peste des petits ruminants (Version 3.0)*. Australian Veterinary Emergency Plan (AUSVETPLAN). Primary Industries Ministerial Council, Canberra, Australia.

Aruni, A.W., Lalitha, P.S., Mohan, A.C., Chitravelu, P. and Anbumani, S.P. (1998) Histopathological study of a natural outbreak of peste des petitis ruminants in goats of Tamilnadu. *Small Ruminants Research* 28, 233–240.

Atmaca, H.T. and Kul, O. (2012) Examination of epithelial tissue cytokine response to natural peste des petits ruminants virus (PPRV) infection in sheep and goats by immunohistochemistry. *Histology and Histopathology* 27, 69–78.

Atta-Ur-Rahman, Ashfaque, M., Rahman, S.U., Akhtar, M. and Ullah, S. (2004) Peste des petits ruminants antigen in mesenteric lymph nodes of goats slaughtered at D.I. Khan. *Pakistan Veterinary Journal* 24, 159–160.

Awa, D.N., Njoya, A. and Ngo Tama, A.C. (2000) Economics of prophylaxis against peste des petits ruminants and gastrointestinal helminthosis in small ruminants in north Cameroon. *Tropical Animal Health and Production* 32, 391–403.

Bailey, D., Banyard, A., Dash, P., Ozkul, A. and Barrett, T. (2005) Full genome sequence of peste des petits ruminants virus, a member of the Morbillivirus genus. *Virus Research* 110, 119–124.

Bailey, D., Chard, L.S., Dash, P., Barrett, T. and Banyard, A.C. (2007) Reverse genetics for peste-des-petits-ruminants virus (PPRV): promoter and protein specificities. *Virus Research* 126, 250–255.

Balamurugan, V., Sen, A., Venkatesan, G., Yadav, V., Bhanot, V., Riyesh, T., Bhanuprakash, V. and Singh, R.K. (2010) Sequence and phylogenetic analyses of the structural genes of virulent isolates and vaccine strains of peste des petits ruminants virus from India. *Transboundary and Emerging Diseases* 57, 352–364.

Balamurugan, V., Sen, A., Venkatesan, G., Bhanot, V., Yadav, V., Bhanuprakash, V. and Singh, R.K. (2012a) Peste des petits ruminants virus detected in tissues from an Asiatic lion (*Panthera leo persica*) belongs to Asian lineage IV. *Journal of Veterinary Science* 13, 203–206.

Balamurugan, V., Sen, A., Venkatesan, G., Rajak, K.K., Bhanuprakash, V. and Singh, R.K. (2012b) Study on passive immunity: time of vaccination in kids born to goats vaccinated against Peste des petits ruminants. *Virologica Sinica* 27, 228–233.

Bandyopadhyay, S.K. (2002) The economic appraisal of PPR control in India. In: *14th Annual Conference and National Seminar on Management of Viral Diseases with Emphasis on Global Trade and WTO Regime*. Indian Virological Society, New Delhi, India.

Banyard, A.C., Parida, S., Batten, C., Oura, C., Kwiatek, O. and Libeau, G. (2010) Global distribution of peste des petits ruminants virus and prospects for improved diagnosis and control. *Journal of General Virology* 91, 2885–2897.

Baron, M.D. and Barrett, T. (1997) Rescue of rinderpest virus from cloned cDNA. *Journal of Virology* 71, 1265–1271.

Baron, M.D. and Barrett, T. (2000) Rinderpest viruses lacking the C and V proteins show specific defects in growth and transcription of viral RNAs. *Journal of Virology* 74, 2603–2611.

Baron, M.D., Parida, S. and Oura, C.A. (2011) Peste des petits ruminants: a suitable candidate for eradication? *Veterinary Record* 169, 16–21.

Barrett, T., Ashley, C.B. and Diallo, A. (2006) Molecular biology of the morbilliviruses. In: Taylor, W.P., Barrett, T. and Pastoret, P.-P. (eds) *Rinderpest and Peste des Petits Ruminants Virus Plagues of Large and Small Ruminants*. Elsevier, Academic Press, London, pp. 31–67.

Bazarghani, T.T., Charkhkar, S., Doroudi, J. and Bani Hassan, E. (2006) A review on peste des petits ruminants (PPR) with special reference to PPR in Iran. *Journal of Veterinary Medicine Series B* 53 (Suppl 1), 17–18.

Beckford, A.P., Kaschula, R.O. and Stephen, C. (1985) Factors associated with fatal cases of measles. A retrospective autopsy study. *South African Medicine Journal* 68, 858–863.

Berhe, G., Minet, C., Le Goff, C., Barrett, T., Ngangnou, A., Grillet, C., Libeau, G., Fleming, M., Black, D.N. and Diallo, A. (2003) Development of a dual recombinant vaccine to protect small ruminants against peste-des-petits-ruminants virus and capripoxvirus infections. *Journal of Virology* 77, 1571–1577.

Bhaskar, A., Bala, J., Varshney, A. and Yadava, P. (2011) Expression of measles virus nucleoprotein induces apoptosis and modulates diverse functional proteins in cultured mammalian cells. *PLoS ONE* 6, e18765.

Bhella, D., Ralph, A. and Yeo, R.P. (2004) Conformational flexibility in recombinant measles virus nucleocapsids visualised by cryo-negative stain electron microscopy and real-space helical reconstruction. *Journal of Molecular Biology* 340, 319–331.

Blom, N., Gammeltoft, S. and Brunak, S. (1999) Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *Journal of Molecular Biology* 294, 1351–1362.

Bodjo, S.C., Couacy-Hymann, E., Koffi, M.Y. and Danho, T. (2006) Assessment of the duration of maternal antibodies specific to the homologous peste des petits ruminant vaccine 'Nigeria 75/1' in Djallonké lambs. *Biokemistri* 18, 99–103.

Bodjo, S.C., Lelenta, M., Couacy-Hymann, E., Kwiatek, O., Albina, E., Gargani, D., Libeau, G. and Diallo, A. (2008) Mapping the Peste des Petits Ruminants virus nucleoprotein: identification of two domains involved in protein self-association. *Virus Research* 131, 23–32.

Bonniwell, M.A. (1980) The use of tissue culture rinderpest vaccine to protect sheep and goats against peste des petits ruminants in the Ashanti region of Ghana. *Bulletin de l'Office Internationale des Epizooties* 92, 1233–1238.

Bourdin, P., Rioche, M. and Laurent, A. (1970) Emploi d'un vaccin antibovipestique produit sur cultures cellulaires dans la prophylaxie de la peste des petits ruminants au Dahomey. *Revue d'élevage et de médecine vétérinaire des pays tropicaux* 23, 295–300.

Boxer, E.L., Nanda, S.K. and Baron, M.D. (2009) The rinderpest virus non-structural C protein blocks the induction of type 1 interferon. *Virology* 385, 134–142.

Braide, V.B. (1981) PPR – a review. *World Animal Review* 39, 25–28.

Brindha, K., Raj, G.D., Ganesan, P.I., Thiagarajan, V., Nainar, A.M. and Nachimuthu, K. (2001) Comparison of virus isolation and polymerase chain reaction for diagnosis of peste des petits ruminants. *Acta Virology* 45, 169–172.

Buczkowski, H., Parida, S., Bailey, D., Barrett, T. and Banyard, A.C. (2012) A novel approach to generating morbillivirus vaccines: negatively marking the rinderpest vaccine. *Vaccine* 30, 1927–1935.

Bundza, A., Afshar, A., Dukes, T.W., Myers, D.J., Dulac, G.C. and Becker, S.A. (1988) Experimental peste des petits ruminants (goat plague) in goats and sheep. *Canadian Journal of Veterinary Research* 52, 46–52.

Caignard, G., Bourai, M., Jacob, Y., Tangy, F. and Vidalain, P.O. (2009) Inhibition of IFN-alpha/beta signaling by two discrete peptides within measles virus V protein that specifically bind STAT1 and STAT2. *Virology* 383, 112–120.

Chard, L.S., Bailey, D.S., Dash, P., Banyard, A.C. and Barrett, T. (2008) Full genome sequences of two virulent strains of peste-des-petits ruminants virus, the Cote d'Ivoire 1989 and Nigeria 1976 strains. *Virus Research* 136, 192–197.

Chen, W., Hu, S., Qu, L., Hu, Q., Zhang, Q., Zhi, H., Huang, K. and Bu, Z. (2010) A goat poxvirus-vectored peste-des-petits-ruminants vaccine induces long-lasting neutralization antibody to high levels in goats and sheep. *Vaccine* 28, 4742–4750.

Choi, K.S., Nah, J.J., Ko, Y.J., Kang, S.Y., Yoon, K.J. and Jo, N.I. (2005) Antigenic and immunogenic investigation of B-cell epitopes in the nucleocapsid protein of peste des petits ruminants virus. *Clinical Diagnosis and Laboratory Immunology* 12, 114–121.

Ciancanelli, M.J. and Basler, C.F. (2006) Mutation of YMYL in the Nipah virus matrix protein abrogates budding and alters subcellular localization. *Journal of Virology* 80, 12,070–12,078.

Cosby, S., Kai, C. and Yamanouchi, K. (2005) *Immunology of Rinderpest: An Immunosuppression but a Lifelong Vaccine Protection*. Academic Press, Elsevier, Amsterdam, The Netherlands.

Cosseddu, G.M., Pinoni, C., Polci, A., Sebhatu, T., Lelli, R. and Monaco, F. (2013) Characterization of peste des petits ruminants virus, Eritrea, 2002–2011. *Emerging Infectious Diseases* 19, 160–161.

Couacy-Hymann, E., Bodjo, S.C., Danho, T., Koffi, M.Y., Libeau, G. and Diallo, A. (2007) Early detection of viral excretion from experimentally infected goats with peste-des-petits ruminants virus. *Preventive Veterinary Medicine* 78, 85–88.

Das, S.C., Baron, M.D. and Barrett, T. (2000) Recovery and characterization of a chimeric rinderpest virus with the glycoproteins of peste-des-petits-ruminants virus: homologous F and H proteins are required for virus viability. *Journal of Virology* 74, 9039–9047.

Dhar, P., Sreenivasa, B.P., Barrett, T., Corteyn, M., Singh, R.P. and Bandyopadhyay, S.K. (2002) Recent epidemiology of peste des petits ruminants virus (PPRV). *Veterinary Microbiology* 88, 153–159.

Diallo, A. (2006) Control of peste des petits ruminants and poverty alleviation? *Journal of Veterinary Medicine Series B: Infectous Diseases in Veterinary Public Health* 53 (Suppl 1), 11–13.

Diallo, A., Barrett, T., Lefevre, P.C. and Taylor, W.P. (1987) Comparison of proteins induced in cells infected with rinderpest and peste des petits ruminants viruses. *Journal of General Virology* 68, 2033–2038.

Diallo, A., Taylor, W.P., Lefèvre, P.C. and Provost, A. (1989) Atténuation d'une souche de virus de la peste des petits ruminants: candidat pour un vaccin homologue vivant. *Revue d'élevage et de médecine vétérinaire des pays tropicaux* 42, 311–317.

Diallo, A., Barrett, T., Barbron, M., Meyer, G. and Lefevre, P.C. (1994) Cloning of the nucleocapsid protein gene of peste-des-petits-ruminants virus: relationship to other morbilliviruses. *Journal of General Virology* 75, 233–237.

Diallo, A., Minet, C., Berhe, G., Le Goff, C., Black, D.N., Fleming, M., Barrett, T., Grillet, C. and Libeau, G. (2002) Goat immune response to capripox vaccine expressing the hemagglutinin protein of peste des petits ruminants. *Annals of the New York Academy of Sciences* 969, 88–91.

Diallo, A., Minet, C., Le Goff, C., Berhe, G., Albina, E., Libeau, G. and Barrett, T. (2007) The threat of peste des petits ruminants: progress in vaccine development for disease control. *Vaccine* 25, 5591–5597.

Durojaiye, O.A., Taylor, W.P. and Smale, C. (1985) The ultrastructure of peste des petits ruminants virus. *Zentralblatt Fur Veterinarmedizin Reihe B* 32, 460–465.

El Harrak, M., Touil, N., Loutfi, C., Hammouchi, M., Parida, S., Sebbar, G., Chaffai, N., Harif, B., Messoudi, N., Batten, C. and Oura, C.A. (2012) A reliable and reproducible experimental challenge model for peste des petits ruminants virus. *Journal of Clinical Microbiology* 50, 3738–3740.

Ezeibe, M.C., Okoroafor, O.N., Ngene, A.A., Eze, J.I., Eze, I.C. and Ugonabo, J.A. (2008) Persistent detection of peste de petits ruminants antigen in the faeces of recovered goats. *Tropical Animal Health and Production* 40, 517–519.

Ezeokoli, C.D., Umoh, J.U., Chineme, C.N., Isitor, G.N. and Gyang, E.O. (1986) Clinical and epidemiological features of peste des petits ruminants in Sokoto Red goats. *Revue d'élevage et de médecine vétérinaire des pays tropicaux* 39, 269–273.

Flanagan, E.B., Ball, L.A. and Wertz, G.W. (2000) Moving the glycoprotein gene of vesicular stomatitis virus to promoter-proximal positions accelerates and enhances the protective immune response. *Journal of Virology* 74, 7895–7902.

Galbraith, S.E., Tiwari, A., Baron, M.D., Lund, B.T., Barrett, T. and Cosby, S.L. (1998) Morbillivirus downregulation of CD46. *Journal of Virology* 72, 10,292–10,297.

Galbraith, S.E., McQuaid, S., Hamill, L., Pullen, L., Barrett, T. and Cosby, S.L. (2002) Rinderpest and peste des petits ruminants viruses exhibit neurovirulence in mice. *Journal of Neurovirology* 8, 45–52.

Gargadennec, L. and Lalanne, A. (1942) La peste des petits ruminants. *Bulletin des Services Zoo Technique et des Epizootie de l'Afrique Occidentale Française* 5, 16–21.

Ghosh, S., Parvez, M.K., Banerjee, K., Sarin, S.K. and Hasnain, S.E. (2002) Baculovirus as mammalian cell expression vector for gene therapy: an emerging strategy. *Molecular Therapy* 6, 5–11.

Gibbs, E.P., Taylor, W.P., Lawman, M.J. and Bryant, J. (1979) Classification of peste des petits ruminants virus as the fourth member of the genus Morbillivirus. *Intervirology* 11, 268–274.

Gilbert, Y. and Monnier, J. (1962) Adaptation du virus de la peste des petits ruminants aux cultures cellulaires. *Revue d'élevage et de médecine vétérinaire des pays tropicaux* 15, 321.

Hamdy, F.M., Dardiri, A.H., Nduaka, O., Breese, S.R.J. and Ihemelandu, E.C. (1976) Etiology of the stomatitis pneumcenteritis complex in Nigerian dwarf goats. *Canadian Journal of Comparative Medicine* 40, 276–284.

Hammouchi, M., Loutfi, C., Sebbar, G., Touil, N., Chaffai, N., Batten, C., Harif, B., Oura, C. and El Harrak, M. (2012) Experimental infection of alpine goats with a Moroccan strain of peste des petits ruminants virus (PPRV). *Veterinary Microbiology* 160, 240–244.

Heaney, J., Barrett, T. and Cosby, S.L. (2002) Inhibition of in vitro leukocyte proliferation by morbilliviruses. *Journal of Virology* 76, 3579–3584.

Horikami, S.M., Smallwood, S., Bankamp, B. and Moyer, S.A. (1994) An amino-proximal domain of the L protein binds to the P protein in the measles virus RNA polymerase complex. *Virology* 205, 540–545.

Hu, Q., Chen, W., Huang, K., Baron, M.D. and Bu, Z. (2012) Rescue of recombinant peste des petits ruminants virus: creation of a GFP-expressing virus and application in rapid virus neutralization test. *Veterinary Research* 43, 48.

Ihemelandu, E.C., Nduaka, O. and Ojukwu, E.M. (1985) Hyperimmune serum in the control of peste des petits ruminants. *Tropical Animal Health and Production* 17, 83–88.

Jagtap, S.P., Rajak, K.K., Garg, U.K., Sen, A., Bhanuprakash, V., Sudhakar, S.B., Balamurugan, V., Patel, A., Ahuja, A., Singh, R.K. and Vanamayya, P.R. (2012) Effect of immunosuppression on pathogenesis of peste des petits ruminants (PPR) virus infection in goats. *Microbial Pathogenesis* 52, 217–226.

Johansson, K., Bourhis, J.M., Campanacci, V., Cambillau, C., Canard, B. and Longhi, S. (2003) Crystal structure of the measles virus phosphoprotein domain responsible for the induced folding of the C-terminal domain of the nucleoprotein. *Journal of Biological Chemistry* 278, 44,567–44,573.

Jones, L., Giavedoni, L., Saliki, J.T., Brown, C., Mebus, C. and Yilma, T. (1993) Protection of goats against peste des petits ruminants with a vaccinia virus double recombinant expressing the F and H genes of rinderpest virus. *Vaccine* 11, 961–964.

Keita, D., Servan de Almeida, R., Libeau, G. and Albina, E. (2008) Identification and mapping of a region on the mRNA of Morbillivirus nucleoprotein susceptible to RNA interference. *Antiviral Research* 80, 158–167.

Khandelwal, A., Renukaradhya, G.J., Rajasekhar, M., Sita, G.L. and Shaila, M.S. (2011) Immune responses to hemagglutinin-neuraminidase protein of peste des petits ruminants virus expressed in transgenic peanut plants in sheep. *Veterinary Immunology and Immunopathology* 140, 291–296.

Kul, O., Kabakci, N., Atmaca, H.T. and Ozkul, A. (2007) Natural peste des petits ruminants virus infection: novel pathologic findings resembling other morbillivirus infections. *Veterinary Pathology* 44, 479–486.

Kul, O., Kabakci, N., Ozkul, A., Kalender, H. and Atmaca, H.T. (2008) Concurrent peste des petits ruminants virus and pestivirus infection in stillborn twin lambs. *Veterinary Pathology* 45, 191–196.

Kulkarni, D.D., Bhikane, A.U., Shaila, M.S., Varalakshmi, P., Apte, M.P. and Narladkar, B.W. (1996) Peste des petits ruminants in goats in India. *Veterinary Record* 138, 187–188.

Kumar, A., Singh, S.V., Rana, R., Vaid, R.K., Misri, J. and Vs, V. (2001) PPR outbreak in goats: epidemiological and therapeutic studies. *Indian Journal of Animal Sciences* 71, 815–818.

Kwiatek, O., Minet, C., Grillet, C., Hurard, C., Carlsson, E., Karimov, B., Albina, E., Diallo, A. and Libeau, G. (2007) Peste des petits ruminants (PPR) outbreak in Tajikistan. *Journal of Comparative Pathology* 136, 111–119.

Kwiatek, O., Ali, Y.H., Saeed, I.K., Khalafalla, A.I., Mohamed, O.I., Obeida, A.A., Abdelrahman, M.B., Osman, H.M., Taha, K.M., Abbas, Z., El Harrak, M., Lhor, Y., Diallo, A., Lancelot, R., Albina, E. and Libeau, G. (2011) Asian lineage of peste des petits ruminants virus, Africa. *Emerging Infectious Diseases* 17, 1223–1231.

Laine, D., Trescol-Biemont, M.C., Longhi, S., Libeau, G., Marie, J.C., Vidalain, P.O., Azocar, O., Diallo, A., Canard, B., Rabourdin-Combe, C. and Valentin, H. (2003) Measles virus (MV) nucleoprotein binds to a novel cell surface receptor distinct from FcgammaRII via its C-terminal domain: role in MV-induced immunosuppression. *Journal of Virology* 77, 11,332–11,346.

Lamb, A. and Kolakofsky, D. (2001) *Paramyxoviridae: the viruses and their replication. Fields Virology*. Lippincott Williams & Wilkins, Philadelphia, Pennsylvania.

Langedijk, J.P., Daus, F.J. and Van Oirschot, J.T. (1997) Sequence and structure alignment of Paramyxoviridae attachment proteins and discovery of enzymatic activity for a morbillivirus hemagglutinin. *Journal of Virology* 71, 6155–6167.

Lefevre, P.C. and Diallo, A. (1990) Peste des petits ruminants. *Revue Scientifique et Technique* 9, 935–981.

Lefevre, P.C., Diallo, A., Schenkel, F., Hussein, S. and Staak, G. (1991) Serological evidence of peste des petits ruminants in Jordan. *Veterinary Record* 128, 110.

Libeau, G., Diallo, A., Calvez, D. and Lefevre, P.C. (1992) A competitive ELISA using anti-N monoclonal antibodies for specific detection of rinderpest antibodies in cattle and small ruminants. *Veterinary Microbiology* 31, 147–160.

Libeau, G., Diallo, A., Colas, F. and Guerre, L. (1994) Rapid differential diagnosis of rinderpest and peste des petits ruminants using an immunocapture ELISA. *Veterinary Record* 134, 300–304.

Libeau, G., Prehaud, C., Lancelot, R., Colas, F., Guerre, L., Bishop, D.H. and Diallo, A. (1995) Development of a competitive ELISA for detecting antibodies to the peste des petits ruminants virus using a recombinant nucleoprotein. *Research in Veterinary Science* 58, 50–55.

Luka, P.D., Erume, J., Mwiine, F.N. and Ayebazibwe, C. (2012) Molecular characterization of peste des petits ruminants virus from the Karamoja region of Uganda (2007–2008). *Archives of Virology* 157, 29–35.

Mahapatra, M., Parida, S., Egziabher, B.G., Diallo, A. and Barrett, T. (2003) Sequence analysis of the phosphoprotein gene of peste des petits ruminants (PPR) virus: editing of the gene transcript. *Virus Research* 96, 85–98.

Mahapatra, M., Parida, S., Baron, M.D. and Barrett, T. (2006) Matrix protein and glycoproteins F and H of Peste-des-petits-ruminants virus function better as a homologous complex. *Journal of General Virology* 87, 2021–2029.

Martrenchar, A., Zoyem, N. and Diallo, A. (1997) Experimental study of a mixed vaccine against peste des petits ruminants and capripox infection in goats in Northern Cameroon. *Small Ruminant Research* 26, 39–44.

Meng, X., Dou, Y., Zhai, J., Zhang, H., Yan, F., Shi, X., Luo, X., Li, H. and Cai, X. (2011) Tissue distribution and expression of signaling lymphocyte activation molecule receptor to peste des petits ruminant virus in goats detected by real-time PCR. *Journal of Molecular Histology* 42, 467–472.

Meyer, G. and Diallo, A. (1995) The nucleotide sequence of the fusion protein gene of the peste des petits ruminants virus: the long untranslated region in the 5'-end of the F-protein gene of morbilliviruses seems to be specific to each virus. *Virus Research* 37, 23–35.

Mioulet, V., Barrett, T. and Baron, M.D. (2001) Scanning mutagenesis identifies critical residues in the rinderpest virus genome promoter. *Journal of General Virology* 82, 2905–2911.

Mitra-Kaushik, S., Nayak, R. and Shaila, M.S. (2001) Identification of a cytotoxic T-cell epitope on the recombinant nucleocapsid proteins of Rinderpest and Peste des petits ruminants viruses presented as assembled nucleocapsids. *Virology* 279, 210–220.

Mondal, B., Sreenivasa, B.P., Dhar, P., Singh, R.P. and Bandyopadhyay, S.K. (2001) Apoptosis induced by peste des petits ruminants virus in goat peripheral blood mononuclear cells. *Virus Research* 73, 113–119.

Mondal, B., Sen, A., Chand, K., Biswas, S.K., De, A., Rajak, K.K. and Chakravarti, S. (2009) Evidence of mixed infection of peste des petits ruminants virus and bluetongue virus in a flock of goats as confirmed by detection of antigen, antibody and nucleic acid of both the viruses. *Tropical Animal Health and Production* 41, 1661–1667.

Moussallem, T.M., Guedes, F., Fernandes, E.R., Pagliari, C., Lancellotti, C.L., De Andrade, H.F. Jr and Duarte, M.I. (2007) Lung involvement in childhood measles: severe immune dysfunction revealed by quantitative immunohistochemistry. *Human Pathology* 38, 1239–1247.

Munir, M. (2013) Role of wild small ruminants in the epidemiology of peste des petits ruminants. *Transboundary and Emerging Diseases* doi: 10.1111/tbed.12052.

Munir, M., Siddique, M. and Ali, Q. (2009) Comparative efficacy of standard AGID and precipitinogen inhibition test with monoclonal antibodies based competitive ELISA for the serology of Peste des Petits Ruminants in sheep and goats. *Tropical Animal Health and Production* 41, 413–420.

Munir, M., Abubakar, M., Zohari, S. and Berg, M. (2012a) Serodiagnosis of Peste des Petits Ruminants Virus. In: Al-Moslih, M. (ed.) *Serological Diagnosis of Certain Human, Animal and Plant Diseases*. InTech, Croatia, pp. 37–58.

Munir, M., Zohari, S., Saeed, A., Khan, Q.M., Abubakar, M., Leblanc, N. and Berg, M. (2012b) Detection and phylogenetic analysis of peste des petits ruminants virus isolated from outbreaks in Punjab, Pakistan. *Transboundary and Emerging Diseases* 59, 85–93.

Munir, M., Zohari, S., Suluku, R., Leblanc, N., Kanu, S., Sankoh, F.A., Berg, M., Barrie, M.L. and Stahl, K. (2012c) Genetic characterization of peste des petits ruminants virus, Sierra Leone. *Emerging Infectious Diseases* 18, 193–195.

Munir, M., Zohari, S. and Berg, M. (2013) Current advances in molecular diagnosis and vaccines for peste des petits ruminants. In: Munir, M., Zohari, S. and Berg, M. (eds) *Molecular Biology and Pathogenesis of Peste des Petits Ruminants Virus*. Springer, Berlin, Germany, pp. 105–133.

Murphy, S.K. and Parks, G.D. (1999) RNA replication for the paramyxovirus simian virus 5 requires an internal repeated (CGNNNN) sequence motif. *Journal of Virology* 73, 805–809.

Muthuchelvan, D., Sanyal, A., Singh, R.P., Hemadri, D., Sen, A., Sreenivasa, B.P., Singh, R.K. and Bandyopadhyay, S.K. (2005) Comparative sequence analysis of the large polymerase protein (L) gene of peste-des-petits ruminants (PPR) vaccine virus of Indian origin. *Archive of Virology* 150, 2467–2481.

Nahed, A.K., Hanan, S.A., Hanan, M.S., Laila, A.S. and Fatma, S.M. (2004) The production and evaluation of a standard diagnostic peste des petits ruminants (PPR) hyperimmune serum prepared from the Egyptian antigen (Egypt 87). *Egyptian Journal of Immunology* 11, 9–14.

Nanda, Y.P., Chatterjee, A., Purohit, A.K., Diallo, A., Innui, K., Sharma, R.N., Libeau, G., Thevasagayam, J.A., Bruning, A., Kitching, R.P., Anderson, J., Barrett, T. and Taylor, W.P. (1996) The isolation of peste des petits ruminants virus from northern India. *Veterinary Microbiology* 51, 207–216.

Obi, T.U., Ojo, M.O., Durojaiye, O.A., Kasali, O.B., Akpavie, S. and Opasina, D.B. (1983) Peste des petits ruminants (PPR) in goats in Nigeria: clinical, microbiological and pathological features. *Zentralblatt Für Veterinarmedizin B* 30, 751–761.

Opal, S.M. and Depalo, V.A. (2000) Anti-inflammatory cytokines. *Chest* 117, 1162–1172.

Parida, S., Mahapatra, M., Kumar, S., Das, S.C., Baron, M.D., Anderson, J. and Barrett, T. (2007) Rescue of a chimeric rinderpest virus with the nucleocapsid protein derived from peste-des-petits-ruminants virus: use as a marker vaccine. *Journal of General Virology* 88, 2019–2027.

Patel, A., Rajak, K.K., Balamurugan, V., Sen, A., Sudhakar, S.B., Bhanuprakash, V., Singh, R.K. and Pandey, A.B. (2012) Cytokines expression profile and kinetics of peste des petits ruminants virus antigen and antibody in infected and vaccinated goats. *Virologica Sinica* 27, 265–271.

Patterson, J.B., Thomas, D., Lewicki, H., Billeter, M.A. and Oldstone, M.B. (2000) V and C proteins of measles virus function as virulence factors in vivo. *Virology* 267, 80–89.

Pawar, R.M., Raj, G.D., Kumar, T.M., Raja, A. and Balachandran, C. (2008) Effect of siRNA mediated suppression of signaling lymphocyte activation molecule on replication of peste des petits ruminants virus in vitro. *Virus Research* 136, 118–123.

Perry, B.D., Randolph, T.F., McDermott, J.J., Sones, K.R. and Thornton, P.K. (2002) *Investing in Animal Health Research to Alleviate Poverty*. International Livestock Research Institute, Nairobi, Kenya.

Plemper, R.K., Hammond, A.L. and Cattaneo, R. (2001) Measles virus envelope glycoproteins hetero-oligomerize in the endoplasmic reticulum. *Journal of Biological Chemistry* 276, 44,239–44,246.

Qin, J., Huang, H., Ruan, Y., Hou, X., Yang, S., Wang, C., Huang, G., Wang, T., Feng, N., Gao, Y. and Xia, X. (2012) A novel recombinant Peste des petits ruminants–canine adenovirus vaccine elicits long-lasting neutralizing antibody response against PPR in goats. *PLoS ONE* 7, e37170.

Raghavendra, L., Setty, D.R.L. and Raghavan, R. (1997) (2000) Clinico-biochemical changes in sheep and goats experimentally infected with Vero cell adapted peste-des petits ruminants (PPR) virus. *International Journal of Animal Sciences* 15, 171–173.

Rahaman, A., Srinivasan, N., Shamala, N. and Shaila, M.S. (2003) The fusion core complex of the peste des petits ruminants virus is a six-helix bundle assembly. *Biochemistry* 42, 922–931.

Rahman, M.M., Shaila, M.S. and Gopinathan, K.P. (2003) Baculovirus display of fusion protein of peste des petits ruminants virus and hemagglutination protein of rinderpest virus and immunogenicity of the displayed proteins in mouse model. *Virology* 317, 36–49.

Rajak, K.K., Sreenivasa, B.P., Hosamani, M., Singh, R.P., Singh, S.K., Singh, R.K. and Bandyopadhyay, S.K. (2005) Experimental studies on immunosuppressive effects of peste des petits ruminants (PPR) virus in goats. *Comparative Immunology, Microbiology and Infectious Diseases* 28, 287–296.

Renukaradhya, G.J., Sinnathamby, G., Seth, S., Rajasekhar, M. and Shaila, M.S. (2002) Mapping of B-cell epitopic sites and delineation of functional domains on the hemagglutinin-neuraminidase protein of peste des petits ruminants virus. *Virus Research* 90, 171–185.

Riedl, P., Moll, M., Klenk, H.D. and Maisner, A. (2002) Measles virus matrix protein is not cotransported with the viral glycoproteins but requires virus infection for efficient surface targeting. *Virus Research* 83, 1–12.

Romero, C.H., Barrett, T., Chamberlain, R.W., Kitching, R.P., Fleming, M. and Black, D.N. (1994) Recombinant capripoxvirus expressing the hemagglutinin protein gene of rinderpest virus: protection of cattle against rinderpest and lumpy skin disease viruses. *Virology* 204, 425–429.

Romero, C.H., Barrett, T., Kitching, R.P., Bostock, C. and Black, D.N. (1995) Protection of goats against peste des petits ruminants with recombinant capripoxviruses expressing the fusion and haemagglutinin protein genes of rinderpest virus. *Vaccine* 13, 36–40.

Rossiter, P.B. (2004) Peste des petits ruminants. In: Coetzer, J.A.W. and Tustin, R.C. (eds) *Infectious Diseases of Livestock*, 2nd edn. Oxford University Press, Cape Town, South Africa, pp. 660–672.

Rossiter, P.B. and Taylor, W.P. (1994) Peste des petits ruminants. In: Coetzer, J.A.W., Thomson, G.R. and Tustin, R.C. (eds) *Infectious Diseases of Livestock*, vol. 2. Oxford University Press, Cape Town, South Africa.

Roulston, A., Marcellus, R.C. and Branton, P.E. (1999) Viruses and apoptosis. *Annual Review of Microbiology* 53, 577–628.

Sahinduran, S., Albay, M.K., Sezer, K., Ozmen, O., Mamak, N., Haligur, M., Karakurum, C. and Yildiz, R. (2012) Coagulation profile, haematological and biochemical changes in kids naturally infected with peste des petits ruminants. *Tropical Animal Health and Production* 44, 453–457.

Saliki, J.T., Libeau, G., House, J.A., Mebus, C.A. and Dubovi, E.J. (1993) Monoclonal antibody-based blocking enzyme-linked immunosorbent assay for specific detection and titration of peste-des-petits-ruminants virus antibody in caprine and ovine sera. *Journal of Clinical Microbiology* 31, 1075–1082.

Saliki, J.T., House, J.A., Mebus, C.A. and Dubovi, E.J. (1994) Comparison of monoclonal antibody-based sandwich enzyme-linked immunosorbent assay and virus isolation for detection of peste des petits ruminants virus in goat tissues and secretions. *Journal of Clinical Microbiology* 32, 1349–1353.

Saravanan, P., Balamurugan, V., Sen, A., Sarkar, J., Sahay, B., Rajak, K.K., Hosamani, M., Yadav, M.P. and Singh, R.K. (2007) Mixed infection of peste des petits ruminants and orf on a goat farm in Shahjahanpur, India. *Veterinary Record* 160, 410–412.

Saravanan, P., Sen, A., Balamurugan, V., Rajak, K.K., Bhanuprakash, V., Palaniswami, K.S., Nachimuthu, K., Thangavelu, A., Dhinakarraj, G., Hegde, R. and Singh, R.K. (2010) Comparative efficacy of peste des petits ruminants (PPR) vaccines. *Biologicals* 38, 479–485.

Schnell, M.J., Mebatsion, T. and Conzelmann, K.K. (1994) Infectious rabies viruses from cloned cDNA. *EMBO Journal* 13, 4195–4203.

Schnorr, J.J., Seufert, M., Schlender, J., Borst, J., Johnston, I.C., Ter Meulen, V. and Schneider-Schaulies, S. (1997) Cell cycle arrest rather than apoptosis is associated with measles virus contact-mediated immunosuppression in vitro. *Journal of General Virology* 78, 3217–3226.

Sen, A., Saravanan, P., Balamurugan, V., Rajak, K.K., Sudhakar, S.B., Bhanuprakash, V., Parida, S. and Singh, R.K. (2010) Vaccines against peste des petits ruminants virus. *Expert Review of Vaccines* 9, 785–796.

Servan De Almeida, R., Keita, D., Libeau, G. and Albina, E. (2007) Control of ruminant morbillivirus replication by small interfering RNA. *Journal of General Virology* 88, 2307–2311.

Seth, S. and Shaila, M.S. (2001) The hemagglutinin-neuraminidase protein of peste des petits ruminants virus is biologically active when transiently expressed in mammalian cells. *Virus Research* 75, 169–177.

Shaila, M.S., Shamaki, D., Forsyth, M.A., Diallo, A., Goatley, L., Kitching, R.P. and Barrett, T. (1996) Geographic distribution and epidemiology of peste des petits ruminants virus. *Virus Research* 43, 149–153.

Shankar, H., Gupta, V.K. and Singh, N. (1998) Occurrence of peste des petits ruminants like diseases in small ruminants in Uttar Pradesh. *Indian Journal of Animal Sciences* 68, 38–40.

Singh, R.P., Sreenivasa, B.P., Dhar, P., Shah, L.C. and Bandyopadhyay, S.K. (2004) Development of a monoclonal antibody based competitive-ELISA for detection and titration of antibodies to peste des petits ruminants (PPR) virus. *Veterinary Microbiology* 98, 3–15.

Sinnathamby, G., Renukaradhya, G.J., Rajasekhar, M., Nayak, R. and Shaila, M.S. (2001) Immune responses in goats to recombinant hemagglutinin-neuraminidase glycoprotein of Peste des petits ruminants virus: identification of a T cell determinant. *Vaccine* 19, 4816–4823.

Sinnathamby, G., Seth, S., Nayak, R. and Shaila, M.S. (2004) Cytotoxic T cell epitope in cattle from the attachment glycoproteins of rinderpest and peste des petits ruminants viruses. *Viral Immunology* 17, 401–410.

Srinivas, R.P. and Gopal, T. (1996) Peste des petits ruminants (PPR): a new menace to sheep and goats. *Livestock Advisor* 21, 22–26.

Stem, C. (1993) An economic analysis of the prevention of peste des petits ruminants in Nigerian goats. *Preventive Veterinary Medicine* 16, 141–150.

Svitek, N. and Von Messling, V. (2007) Early cytokine mRNA expression profiles predict Morbillivirus disease outcome in ferrets. *Virology* 362, 404–410.

Tao, C., Li, G., Wang, Y. and Huang, H. (2012) Enzymatic reporting of peste des petits ruminants virus genes ligating two specific probes on nanoparticles. *Biotechnology Letters* 35, 613–618.

Taylor, W.P. (1979) Protection of goats against peste-des-petits-ruminants with attenuated rinderpest virus. *Research in Veterinary Science* 27, 321–324.

Taylor, W.P. and Abegunde, A. (1979) The isolation of peste des petits ruminants virus from Nigerian sheep and goats. *Research in Veterinary Science* 26, 94–96.

Taylor, W.P., Al Busaidy, S. and Barrett, T. (1990) The epidemiology of peste des petits ruminants in the Sultanate of Oman. *Veterinary Microbiology* 22, 341–352.

Thombare, N.N. and Sinha, M.K. (2009) Economic implications of Peste des petits ruminants (PPR) disease in sheep and goats: a sample analysis of district Pune, Maharastra. *Agricultural Economics Research Review* 22, 319–322.

Varsanyi, T.M., Utter, G. and Norrby, E. (1984) Purification, morphology and antigenic characterization of measles virus envelope components. *Journal of General Virology* 65, 355–366.

Waret-Szkuta, A., Roger, F., Chavernac, D., Yigezu, L., Libeau, G., Pfeiffer, D.U. and Guitian, J. (2008) Peste des petits ruminants (PPR) in Ethiopia: analysis of a national serological survey. *BMC Veterinary Research* 4, 34.

Woo, P.C., Lau, S.K., Wong, B.H., Fan, R.Y., Wong, A.Y., Zhang, A.J., Wu, Y., Choi, G.K., Li, K.S., Hui, J., Wang, M., Zheng, B.J., Chan, K.H. and Yuen, K.Y. (2012) Feline morbillivirus, a previously undescribed

paramyxovirus associated with tubulointerstitial nephritis in domestic cats. *Proceedings of the National Academy of Sciences of the USA* 109, 5435–5440.

Wosu, L.O. (1994) Current status of peste des petits ruminants (PPR) disease in small ruminants. A review article. *Stud. Research in Veterinary Medicine* 2, 83–90.

Yarim, G.F., Nisbet, C., Yazıcı, Z. and Gumusova, S.O. (2006) Elevated serum total sialic acid concentrations in sheep with peste des petits ruminants. *Medycyna Weterynaryjna* 62, 1375–1377.

Yener, Z., Saglam, Y.S., Temur, A. and Keles, H. (2004) Immunohistochemical detection of peste des petits ruminants viral antigens in tissues from cases of naturally occurring pneumonia in goats. *Small Ruminant Research* 51, 273–277.

Yoneda, M., Bandyopadhyay, S.K., Shiotani, M., Fujita, K., Nuntaprasert, A., Miura, R., Baron, M.D., Barrett, T. and Kai, C. (2002) Rinderpest virus H protein: role in determining host range in rabbits. *Journal of General Virology* 83, 1457–1463.

Yoneda, M., Miura, R., Barrett, T., Tsukiyama-Kohara, K. and Kai, C. (2004) Rinderpest virus phosphoprotein gene is a major determinant of species-specific pathogenicity. *Journal of Virology* 78, 6676–6681.

Yunus, M. and Shaila, M.S. (2012) Establishment of an in vitro transcription system for peste des petits ruminant virus. *Virology Journal* 9, 302.

Zhang, X., Glendening, C., Linke, H., Parks, C.L., Brooks, C., Udem, S.A. and Oglesbee, M. (2002) Identification and characterization of a regulatory domain on the carboxyl terminus of the measles virus nucleocapsid protein. *Journal of Virology* 76, 8737–8746.

6 Rinderpest Virus

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6.1 Introduction

Rinderpest (from the German 'cattle plague') was a disease of significant veterinary importance affecting all species of the even-toed ungulates (*Artiodactyla*). The host range included cattle (both *Bos taurus* and *Bos indicus*), but also extended to small ruminants and exotic wildlife, including buffalo, giraffe, deer, kudu, wildebeest and African warthog. The geographical distribution of rinderpest reflected the history of the disease. Outbreaks attributed to the disease were first recorded in the 4th century in Asia, from which it spread to Europe by movement and transportation of cattle and eventually, in the 19th century, to Africa by colonization with concomitant introduction of domestic cattle. By the 20th century, it was endemic in Africa, Asia, the Middle East and the Indian subcontinent. Rinderpest was one of the most economically devastating veterinary diseases. The severity of a European epidemic in the 18th century led to the establishment of the first veterinary college (in Lyon in 1761) as a direct response to control rinderpest by training people as veterinarians. The Great African Pandemic in 1885 alone caused mortality rates of up to 90% in both domestic stock and wildlife, and nearly led to the extinction of the Masai people. A second epidemic from 1979 to 1983 caused the death of over 1 million cattle and it has been estimated that within this period in Nigeria alone more than 500,000 cattle died, causing an economic loss of US$1.9 billion (FAO, 2002).

In response to the ongoing rinderpest outbreaks in Africa, the Pan African Rinderpest Campaign (PARC) for the control of the disease was initiated. In 1984, the United Nation's (UN) Food and Agricultural Organization (FAO) launched the Global Rinderpest Eradication Programme (GREP) with the aim of promoting international coordination and the global eradication of rinderpest, verifying freedom from rinderpest and providing technical guidance to achieve these goals. The availability of a single dose, live attenuated vaccine ('the Plowright vaccine') that could induce lifelong immunity and the development of appropriate companion diagnostic assays were key factors in the eradication of rinderpest. Serological surveillance following vaccination campaigns using standardized assays (originally an indirect ELISA and subsequently a competitive monoclonal antibody (mAb)-based ELISA) allowed the testing of 1000s of samples in the regional laboratories alongside support from national laboratories across Africa, the Middle East and Asia. Furthermore, support was also available for both technology transfer and

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training from the World Reference Laboratories within the EU. Technological developments in diagnostic tools led to the replacement of the indirect ELISA for seromonitoring with an advanced mAb-based competitive ELISA with greatly increased sensitivity, specificity, reproducibility and standardization. Epidemiological studies were supported by the development of technologies such as genetic typing of viruses by reverse transcription PCR, allowing the molecular characterization of virus isolates, and further lineage differentiation enabled assessment of geographical origins of new outbreaks (Forsyth and Barrett, 1995). A rapid diagnostic chromatographic strip test for the pen-side diagnosis of rinderpest, crucial in the final stages of GREP, was also developed. Based on this and through concerted efforts by the participating members of GREP, the programme finally came to fruition in 2011 when rinderpest was declared eradicated worldwide (FAO, 2011; Roeder 2011a, 2011b).

6.2 Rinderpest Causative Agent

Rinderpest was caused by the rinderpest virus (RPV), a member of the *Morbillivirus* genus. Peste des petits ruminants virus (PPRV), canine distemper virus (CDV), measles virus and phocine distemper virus (PDV) are other members of this genus. Morbillivirus virions are generally spherical and approximately 150–300 nm in diameter. They have a lipid-containing envelope that is derived from the host cell membrane on budding. These lipid envelopes contain spike-like glycoprotein protrusions of approximately 8–12 nm in length, spaced 7–10 nm apart, 'studded' across their surfaces called peplomers. Inside the lipid envelope the virions contain a coiled helical nucleocapsid that is 13–18 nm in diameter and up to 1000 nm in length with a pitch of 5.5–7 nm, depending on the genus (Fig. 6.1). The morbilliviruses are characterized by a single-strand negative-sense RNA genome that is 15,882 nucleotides in length. Each virion contains six structural proteins: the three nucleocapsid-associated proteins (N, an RNA-binding protein; P, a phosphoprotein; L, a large protein with characteristics of a polymerase protein); M, an unglycosylated matrix protein located under the virus envelope; and two glycosylated envelope proteins, namely the fusion (F) and attachment (H) proteins. The two virus-encoded non-structural proteins, C and V, are thought to play roles in the virus replication cycle. These two non-structural proteins may be essential for pathogenesis *in vivo* (Kato *et al.*, 1997; Escoffier *et al.*, 1999), although they can be dispensed with *in vitro* (Baron and Barrett, 2000).

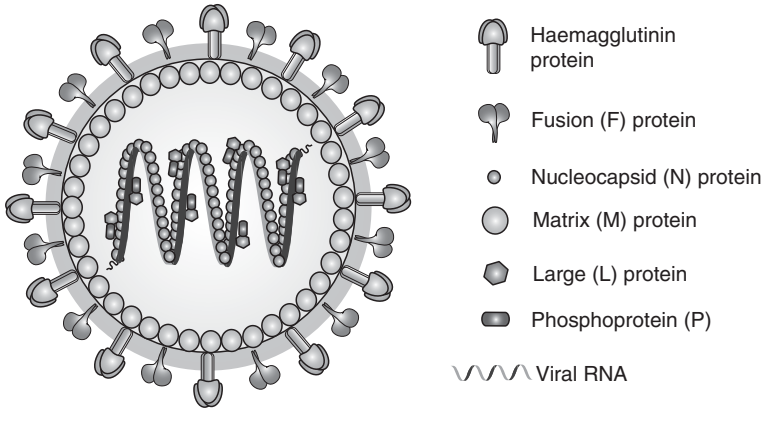

Fig. 6.1. Schematic of the morbillivirus virion.

The structural components of RPV have numerous functions within the virion, including: encapsidation of the nascent genome and antigenome full-length viral RNAs; specific binding of the viral polymerase complex through a conserved domain; formation of a flexible helical nucleocapsid core; and interaction with the viral M protein that is required during assembly of nascent virus particles (Sedlmeier and Neubert, 1998; Curran and Kolakofsky, 1999). The N-terminal 80% of the N protein is relatively well conserved among morbilliviruses and this portion of the protein is thought to form the globular body of the protein.

Of all the morbillivirus proteins, the P protein is antigenically the least conserved (Sheshberadaran *et al.*, 1986) and at the nucleotide level is the most variable gene in the genome. The functioning of the P protein in virus transcription and replication has been inferred from its ability to form distinct complexes with the L and the N proteins during transcription and replication of the genome RNA (Horikami *et al.*, 1992). However, the level of P gene expression seen in infected cells, far in excess of its representation in virions, indicates that as well as functioning in RNA synthesis it is also involved in nucleocapsid assembly.

Importantly, the two non-structural proteins, C and V, are produced from the P open-reading frame (ORF) using alternate mechanisms: C is produced following RNA translation initiation at a second in frame AUG start codon within the P gene, while V is generated following RNA editing within the P ORF to produce a truncated protein. The production of these proteins highlights an evolutionary mechanism by which these viruses maximize coding capacity (Bellini *et al.*, 1985). The morbillivirus C protein has been shown to co-localize with the N, P and L proteins in infected cells, which suggests a role in viral transcription (Sweetman *et al.*, 2001). A recombinant RPV lacking the ability to produce the C protein was found to be defective for growth in a number of cell lines, although the levels of genome and antigenome RNAs produced were comparable to the parent virus. The absence of C from RPV was shown to affect mRNA transcription, suggesting a possible role in initiation, extension or termination of transcription (Baron and Barrett, 2000). A further function identified for the C protein is in counteracting the host response to virus infection through blocking of innate immune responses. A number of paramyxoviruses subvert the host cell's immune response to infection by blocking interferon (IFN) signaling at different stages and in many viral infections this has been attributed to the C protein. The V protein is produced as a result of a pseudotemplated transcription where a G residue is added to the P gene mRNA during transcription (Lamb and Kolakofsky, 2001). In RPV infections, the V protein interacts with free N protein and is distributed throughout the cytoplasm (Baron and Barrett, 2000) and RPV lacking the V protein, in the presence or absence of the C protein, shows greater genome and antigenome production than wildtype virus (Baron and Barrett, 2000). This points to an influence of the V protein on replication rates and may therefore play a role in viral pathogenesis. The role of the accessory proteins of negative strand viruses is reviewed in Gerlier and Lyles (2011).

The M proteins of all the morbilliviruses sequenced to date have a single ORF of 1005 nucleotides (Mahapatra *et al.*, 2003). The differences seen in the lengths of the M genes of morbilliviruses arise from the presence of a long untranslated GC-rich region present at their 5′ ends. These GC-rich regions are of unknown function, but for RPV, removal of this region seems to have little effect on virus growth *in vitro* (T. Barrett, personal communication). Many studies have suggested a role for the M protein in virus budding. In this role the M protein is thought to bring together the RNP complexes, which accumulate in the cytoplasm of the infected cell, and the viral glycoproteins, which, through their synthesis on the rough endoplasmic reticulum (RER), localize to the plasma membrane of the cell (Stricker *et al.*, 1994; Wild *et al.*, 1995). This function is conserved among the negative-stand RNA viruses and within the morbillivirus genus the M gene is the most conserved gene (Limo and Yilma, 1990; Curran *et al.*, 1992; Baron *et al.*, 1994).

The glycoproteins of the morbilliviruses, F and H, are responsible for virus entry into the cell, virus–cell fusion and cell–cell fusion. The RPV F protein is a type I membrane-bound glycoprotein that contains three domains. A short C-terminal cytoplasmic tail, a hydrophobic region of 20–40 residues that anchors the protein in the membrane and an N-terminal domain that extends into the endoplasmic reticulum lumen during synthesis. Interaction between both the F and H proteins of morbilliviruses is required to form an active biological fusion complex (Das *et al.*, 2000; Mahapatra *et al.*, 2006). The H protein is a type II glycoprotein with a single hydrophobic region at its N-terminus that functions both as a signal sequence and anchors the protein to the cell membrane, while the C-terminus extends out into the lumen of the ER or on the external surface of the cell. The H gene displays the highest degree of antigenic variation in the morbilliviruses (Mahy *et al.*, 1988; Blixenkrone-Moller *et al.*, 1996; Bolt *et al.*, 1997) and is the target of neutralizing antibodies (Buczkowski *et al.*, 2012). A common structure is proposed for the H proteins of the paramyxoviruses based on the conservation of various glycine, cysteine and proline residues as well as glycosylation sites (Langedijk *et al.*, 1997; Lamb and Kolakofsky, 2001; Massé *et al.*, 2004; Buczkowski *et al.*, 2012).

The polymerase protein performs a number of different functions during the replication of RPV. It acts as an essential subunit of the viral polymerase complex, through interactions with the P and N proteins. L possesses transcriptase and replicase activities as well as acting to cap, methylate and polyadenylate viral mRNAs. Within the morbilliviruses three highly conserved domains that are separated by two hinge regions have been identified. By incorporating *c-myc* tags at the variable hinge regions of the MeV L protein, it has been shown that these regions vary in their tolerance to insertion and spatial displacement. Abrogation of polymerase function was seen with the insertion of a *c-myc* tag into the N-proximal hinge region that was attributed to the possible reorientation of a catalytic domain. The second hinge region towards the C-terminus of the L gene is more conserved among the morbilliviruses, but insertion of the tag at this position had no effect on polymerase function. Further studies using the green fluorescent protein (GFP) ORF showed that it was possible to insert the entire GFP ORF into the second hinge region without abolishing L protein function. The incorporation of the GFP-containing polymerase into a recombinant virus did not seriously debilitate its growth *in vitro* or *in vivo*. This observation may have many applications for the study of the polymerase unit and virus pathogenicity through the ability to trace the virus in tissues (Duprex *et al.*, 2002; Brown *et al.*, 2005).

6.3 Clinical Signs

Rinderpest was transmitted to susceptible animals via droplet spread through close contact with contaminated nasal/ocular secretions as well as faecal contamination. The incubation period varied between 3 and 15 days, depending on the strain, route of entry, infective dose and herd susceptibility. This prodrome was followed by an acute, febrile period and then by mucopurulent discharge, erosions on the gum and tongue and finally shooting diarrhoea. Necrotic-haemorrhagic erosions in the intestines, enlarged lymph nodes, tachycardia and dehydration were often observed. Death occurred within 6–12 days after the onset of fever. Subacute reactions and atypical forms displayed mild clinical signs and low mortality, whereas peracute reactions characterized by a sudden onset and death (within 2–3 days) were occasionally observed in young calves. Morbidity and mortality rates varied, depending on the virulence of the strain involved, but could be up to 100% in a group of naive animals. Once recovered, the affected animals had generally developed a life-long sterilizing immunity. Small even-toed ungulates such as goats and sheep rarely presented with pyrexia, anorexia and intermittent diarrhoea, but this was species-dependent

and most African goats and sheep exhibited subclinical infections. Rinderpest in European pigs was characterized by pyrexia, conjunctivitis, buccal erosions and death, but in African, Asian and Middle Eastern breeds was subclinical.

6.4 Pathology

Pathological features of rinderpest have been described in detail (Brown and Torres, 1994; Brown *et al.*, 1996; Anderson *et al.*, 1996; Taylor *et al.*, 2005). For the purpose of this chapter, the most common features of rinderpest pathology are summarized here. Perhaps in the most classical form, in cattle and buffalo, rinderpest presented as emaciation and dehydration with signs of diarrhoea and mucopurolent discharge. Congested conjunctiva could be oedematous and corneal ulceration may have occurred. The oral cavity was often affected, but depending on the infecting strain and stage of disease this varied from pinhead or larger necrotic foci to extensive necrosis and gum erosions. The soft palate, pharynx and upper oesophagus might also be affected by necrosis. In some cases, necrosis also occurred in the pillars of the rumen, but with no effect on other areas of the rumen or reticulum. Erosions and haemorrhages might occur in the omasum. The abomasum might also be affected, with evidence of congestion, petechial haemorrhage and oedema. Peyer's patches might show signs of white necrotic foci, but apart from necrosis and sloughing in neighbouring regions, the small intestines usually remained unaffected. The large intestines might be affected by the presence of blood and blood clots in the lumen; intestinal walls might appear congested and affected by oedema and erosions, which often occurred in the upper colon. The characteristic 'tiger or zebra striping' was often seen in the colon and in rectal mucosae in animals with late stage disease. Lungs might be affected by emphysema, congestion and secondary bronchopneumonia. The kidneys were usually unaffected, with some congestion notable in the medulla. However, severe desquamation of the epithelium in the urinary bladder was caused by infiltration of erythrocytes into the underlying stroma.

RPV and other morbilliviruses are highly lymphotrophic and, as such, following RPV infection tissues of the lymphatic system were often severely affected; enlargement of the spleen and lymph nodes were observed with the most damage in the mesenteric lymph nodes and lymphoid tissues associated with the intestines; the gall bladder might be affected by petechiae and ecchymoses. RPV was also epitheliotrophic and so also caused extensive infection within the epithelia of the alimentary, upper respiratory and urogenital tract. Severity of infection and presence at post-mortem findings depended on the strain involved and time of death after the onset of illness. Carcasses of animals that had died early after infection and before the onset of the diarrhoeic phase often presented without the rinderpest-associated soiledness and mucupurolent discharges. Less pronounced post-mortem findings were recorded for sheep and goats, as well as pigs (Fig. 6.2A).

Haematological and biochemical changes were also detected during rinderpest infections. The lymphotrophic nature of RPV caused total leucocyte counts to reduce sharply during the prodromal fever phase of infection and then recovered to normal levels if the animal managed to clear the infection and survive. This drop was attributable to the destruction of both T- and B-lymphcoytes, with a sharp decline during the prodromal fever, which slowly recovered in surviving animals. Research studies demonstrated that RPV grew well in B-cells, CD4+ and CD8+ alpha/beta T-cells, and gamma/delta T cells with no preference for a particular phenotype (Anderson *et al.*, 1996). Monocyte numbers were not affected and neutrophil numbers only appeared affected in fatal cases. Eosinophils appeared to follow the lymphocytic pattern, whereas basophil numbers only dropped in fatal cases after onset of the prodromal fever. Increases of erythrocytes in fatal cases were linked to severe cases of dehydration (Fig. 6.2B).

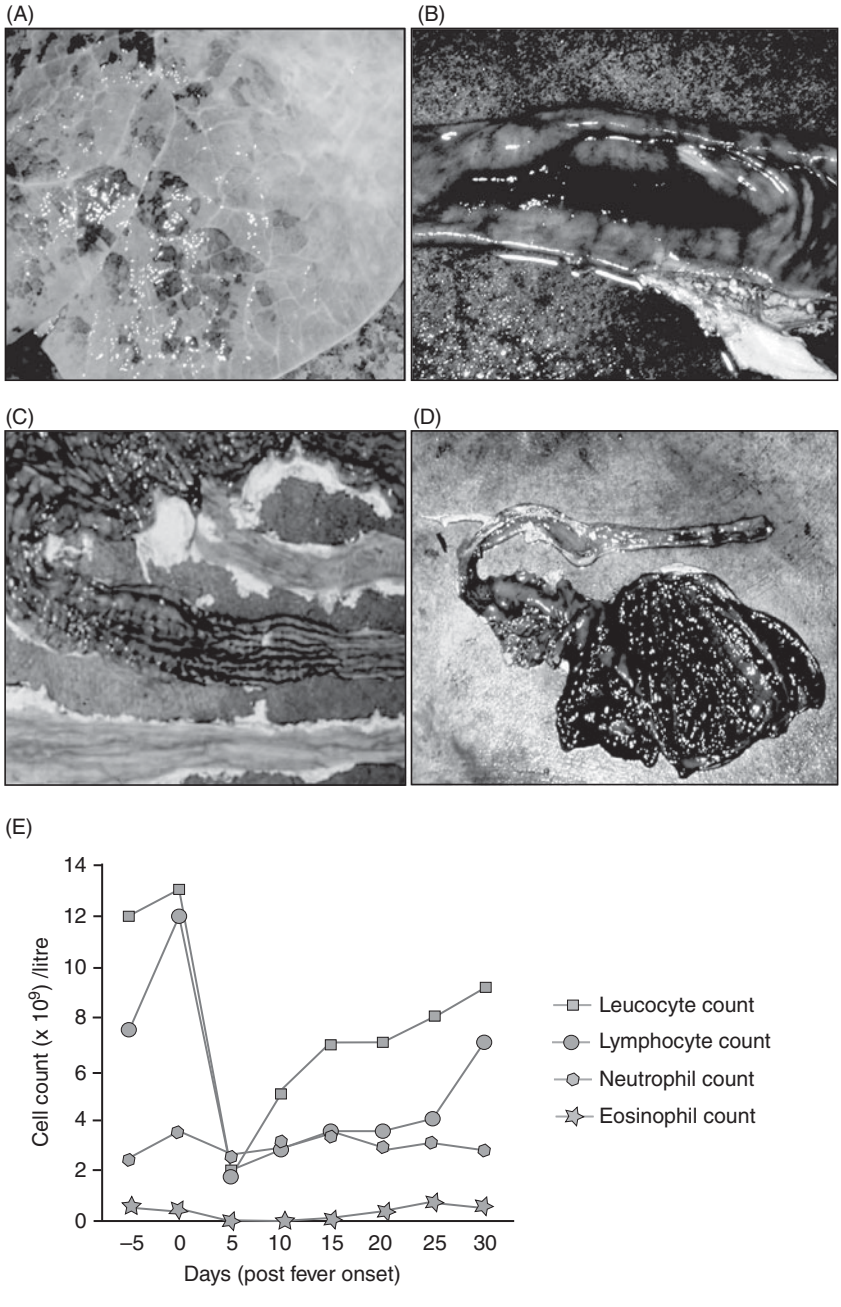

Fig. 6.2. Pathobiology of rinderpest. Characteristic pathological features: (A) emphysema of the lung; (B) inflamed and haemorrhagic serosal and mucosal surfaces of Peyer's patches; (C) zebra striping of the intestines – haemorrhages along the crests of the mucosae gave rise to the characteristic striped pattern, which appeared red when recent and green when older; (D) severe congestion and haemorrhage of the fourth stomach; (E) haematological changes during infection (after Anderson *et al.*, 1996). [(A) courtesy of Dr Ashley Banyard; (B–D) courtesy of Jenny Ryder and Professor John Anderson.]

Various biochemical changes (such as loss of water and electrolytes, metabolic acidosis, hypoglycaemia) were also reported in rinderpest infections (Anderson *et al.*, 1996).

6.5 Diagnosis

Diagnosis of rinderpest took place firstly in the field or at pen-side by thorough examination of suspect animals and carcasses within a herd and the presence of the tell-tale three 'Ds', discharge, diarrhoea and death (Fig. 6.3A). However, many of the clinical signs observed in rinderpest could also be attributable to other viral diseases of livestock, including peste des petits ruminants (PPR), foot and mouth disease (FMD), bovine viral diarrhoea (BVD), Bluetongue (BT) and malignant catarrhal fever (MCF), whose geographical distribution often overlapped with that of RPV. In addition, during the last rinderpest outbreaks in the 21st century, the presence of mild forms of rinderpest made field diagnosis more problematic. For rinderpest to be diagnosed conclusively, it needed to be confirmed in a laboratory. This could be achieved directly by the detection of antigen in the samples from suspect animals, including secretions/excretions and tissues, by the isolation and identification of the strain in question and/or by the detection of specific antibodies in the sera from suspect animals (Fig. 6.3B).

6.5.1 Sample collection for rinderpest antigen detection

For a successful detection of rinderpest antigen in secretions/excretion or tissues, the selection of the right sample and its transportation was crucial. Scientific research had revealed that a high viral load was found in ocular and nasal secretions, gum debris, blood and tissues (including spleen, lymph node and tonsil) while the animal was in the pyrexic stage of the disease. To avoid the rapid dissociation of the virus due to exposure to light, heat and extreme pH, all samples had to be transported on wet ice in a secure vessel such as an icebox. The timing of sampling was crucial. Highest viral loads were found to be present during the prodromal fever and the erosive mucosa phase, with viral shedding decreasing with progression of illness. For sampling to be most effective, it therefore had to take place during the prodromal fever and early onset of the erosive-mucosa phase.

6.5.2 Antigen detection

Direct diagnosis of RPV was achieved in the laboratory by various methods, which can be grouped into classical and new as defined by the methodologies adopted prior to and after the initiation of rinderpest eradication programmes. Classical rinderpest antigen detection included the following methods.

Virus isolation

This method was believed to be the gold standard for the definitive diagnosis of the presence of RPV, especially for regions with previously unknown rinderpest infections. For virus isolation, several prerequisites were vital, including the availability of laboratory facilities with class II laminar flow cabinets, incubators, autoclaves, centrifuges, filtration equipment, microscopes and roller apparatus. Virus isolation was achieved by growing the suspect virus in susceptible cell culture, traditionally – in the case of RPV – primary calf kidney cells. Calf kidney cells had to be established from a donor calf, dissected and processed under sterile conditions to obtain primary cell cultures of actively dividing cells. The cells were eventually grown in roller bottles and placed on roller drums, allowing cells to continue growing without contact inhibition. Primary cell cultures were then used to produce secondary bovine kidney cells for virus isolation. For virus isolation, field specimens

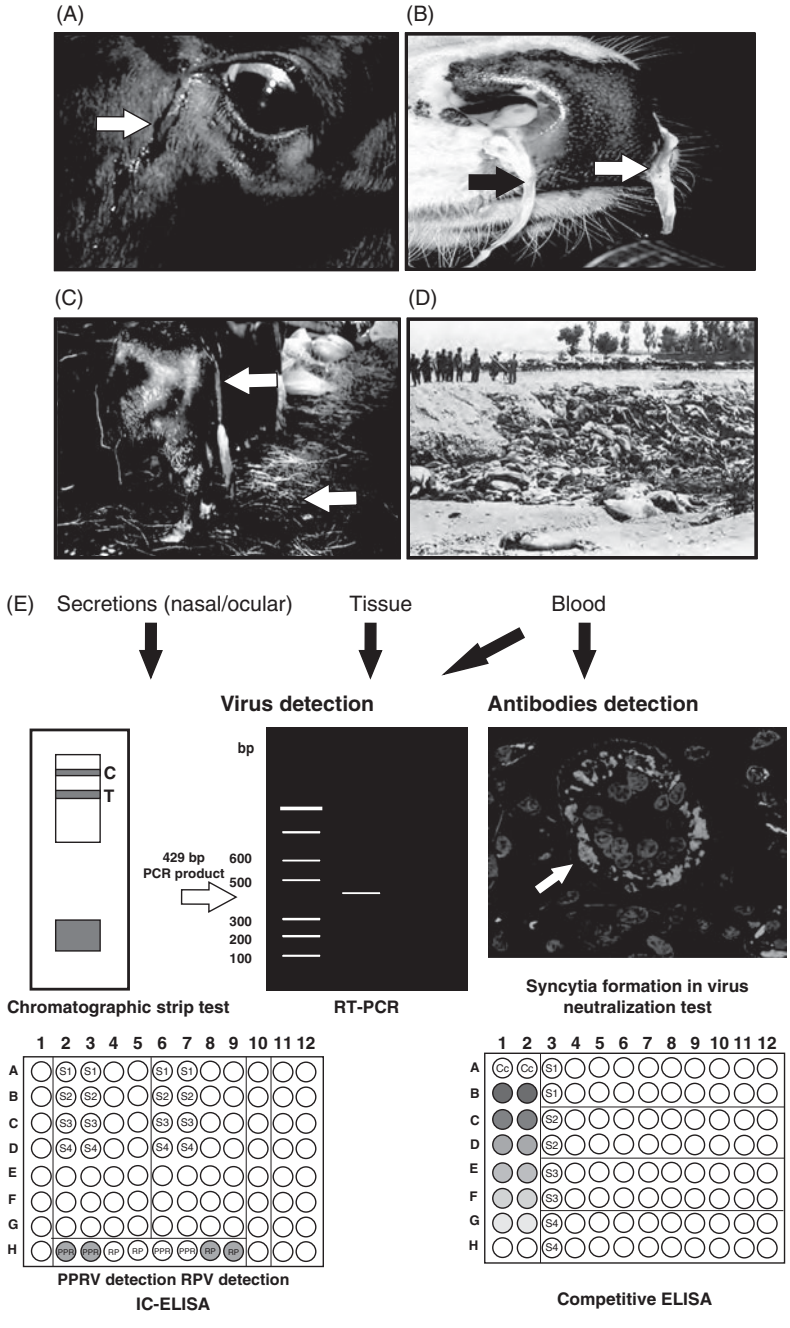

Fig. 6.3. Field and laboratory diagnosis of rinderpest. Field diagnosis based on the '3Ds': (A) ocular and (B) nasal discharge; (C) diarrhoea and (D) death. (E) Laboratory diagnosis: direct by virus detection by chromatographic strip test (also applicable in the field or at pen-side); reverse transcriptase-PCR or immunocapture ELISA. Indirect detection by antibody detection by virus neutralization (arrow points at syncytium formed after infection of cell culture with rinderpest virus and visualized by immunofluorescence) or antibody detection by competitive ELISA. C = control; T = test; bp = base pair; S1 etc. = sample 1 etc.; Cc = control. [(A, B) supplied by Dr Ashley Banyard; (C) supplied by Dr Anke Brüning-Richardson; (D) obtained from FAO.]

including blood and solid tissues were prepared to yield a buffy coat suspension from blood samples and cell suspensions were prepared from ground tissues ready for inoculation into the roller tubes. After inoculation of the samples, the medium was removed from the cell layers and replaced with maintenance medium supplemented with or without rinderpest immune serum. This procedure was carried out over a period of 2 weeks and if no virus was detected by the appearance of rinderpest-specific cytopathic effects (CPE), the cultures were blind passaged and re-examined. Rinderpest CPEs could be identified by the typical appearance of densely granular syncytia in the cultures with no added rinderpest immune serum and by the absence of CPE in the cultures with added immune serum. This type of isolation not only allowed the detection of rinderpest in a sample, but also the generation and harvesting of viral material for further analysis. Drawbacks of this technique included the requirement of tissue culture facilities and highly skilled staff, the time-consuming nature of the test and the fact that PPR caused similar infection in bovine kidney cells, thus preventing a differential diagnosis.

Agar gel immunodiffusion test (AGID)

Agar gel immunodiffusion tests rely on antigen precipitation after interaction with antibody. In the case of RPV detection, rinderpest antigen in a suspect sample was deposited in a well cut into an agar gel and allowed to diffuse within the gel. Upon interaction between antigen in test samples and rinderpest antibody diffusing out from another well located within the same agar gel, a white precipitate visible to the eye was formed. The technique was simple to perform and, with the addition of a control antigen and antibody, easy to interpret. Kits for agar-gel immunodiffusion were readily available and easy to use. Interpretation was straightforward; however, a negative result did not negate a preliminary diagnosis in the field because antigen concentrations in the samples may have been below the sensitivity threshold of the test. In this sense, only a positive result was meaningful. Alongside this, the AGID test was also unable to differentiate between RPV and PPRV.

Direct and indirect immunoperoxidase tests

This method was based on the detection of antigen on slides containing sections of biopsied tissues from suspect animals. Here, antibodies to rinderpest were allowed to bind directly (following labelling with horse-radish peroxidase (HRP)) or indirectly (through the interaction with secondary antibody labelled with HRP) to antigen contained within the sample. Rinderpest antigen was then visualized by a brown coloration of the cytoplasm of infected cells within the tissue by light microscopy. This method had the advantage of producing permanent records, because the stained slides can be stored for reference. Similar results could be achieved by immunofluorescence tests (IF), where smears containing lymph node, liver and kidney samples or coverslips containing cell culture material from suspect animals were fixed with acetone and then reacted with rinderpest-specific antibodies. The antibodies were, again, either directly labelled with a fluorescein-isothiocyanate (FITC) conjugate or could be detected indirectly by the addition of a secondary FITC-conjugated antibody. Instead of detecting cytoplasmic RPV localization by a brown coloration, the presence of RPV was indicated by a green fluorescent cytoplasmic stain using an immunofluorescence microscope (Rossiter and Jessett, 1982). Drawbacks of this technique included the specificity of the antibodies used, which initially were of a polyclonal nature, giving rise to non-specific background staining. However, with the advent of monoclonal antibody technology, more specific mAb preparations were developed. Another drawback for both techniques was the need for sophisticated equipment such as a light or fluorescence microscope in resource-limited settings.

Counter-immunoelectrophoresis (CIEP)

CIEP was a modified version of AGID utilizing electrophoresis. This was based on the observation that antigen preparations migrate towards the anode (+) in a gel when an electric current is applied, whereas antibodies migrate towards the cathode (–). Antibody–antigen interactions were visualized by the development of precipitates between the suspect antigen and antibody wells. This test had been originally designed for RPV detection in parallel by Ali *et al*. (1979) and Rossiter and Mushi (1980), with a rapid diagnosis available after 80 minutes and 40 minutes, respectively. It was further adapted for use in the field by Rossiter (1984) and finally even more improved by Injairu (1984) to be run as a battery-operated kit in the field.

For the rapid and high throughput detection of RPV needed for the global rinderpest eradication programme, several new technologies emerged, which included antigen-detecting ELISA, a rapid chromatographic strip test and RT-PCRs.

RPV antigen detection ELISA

This assay was based on the detection of antigen in a suspect sample by a rinderpest-specific antibody, with the whole assay carried out in a 96-well plastic plate. Libeau *et al*. (1992, 1994) developed a RPV-specific ELISA by trapping suspect antigen with an anti-nucleocapsid (N)-protein mAb cross reactive for both RPV and PPRV, which had been coated onto the wells within the ELISA plate, and then detecting the causative agent by incubating the trapped complex with RPV- and PPRV-specific biotinylated anti-N-protein mAbs. Detection was achieved by incubating the antibody–antigen complexes in the plate with a streptavidin-peroxidase conjugate giving rise to coloration in the wells if RPV was present in the sample. Results were determined by taking colorimetric readings with an ELISA plate reader. Optical density values greater than twice the mean of the blank control were recorded as a positive result. Many different sample types could be used in this system, including ocular, nasal and buccal secretions as well as tissues such as lymph node, lung and spleen. The incubation with two specific antibodies allowed the differential diagnosis of RPV and PPRV. The ELISA was standardized and supplied in kit format by CIRAD/EMVT, Paris, France. Over the years, more mAb-based immunocapture ELISAs were developed, for example an ELISA for the detection of RPV using mAbs against the N-protein (Shah *et al*., 2004).

Rapid chromatographic strip test

When GREP reached its final phase after the completion of mass vaccinations, it was essential that any remaining foci of infection were identified. It became clear that a simple, rapid and robust pen-side test for the detection of RPV in the field was needed. Valuable time for emergency vaccinations could be wasted if samples from a suspect outbreak were to be transported back for a laboratory confirmation with a possible rinderpest outbreak at hand. The format of a rapid chromatographic strip test similar to home pregnancy test kits seemed to be ideal to address this issue. These pen-side tests were: easy to use; required only small amounts of sample to be added directly to the test; rapid (with a result available within 15 minutes); and robust enough to be transported and operated even under extreme climatic conditions. The device was based on the interaction of RPV antigen (in the lachrymal fluid from the suspect animal) and a rinderpest-specific antibody bound onto blue latex particles contained within a pad. Rinderpest-specific antibody was also bound to nitrocellulose encased within the main body of the test device and attached to the sample pad. A result window indicated if a sample was positive for RPV and also confirmed that the test had worked. This test allowed veterinarians to take lachrymal fluid from a suspect animal using a cotton bud, extract any antigen into a supplied buffer solution and then apply this sample directly to the device. Upon contact with the antigen-buffer solution, the air-dried antibodies bound to the blue latex on the sample pad

were rehydrated, interacted with any antigen in the sample and then travelled by capillary action along the nitrocellulose strip. Any antigen–antibody complexes labelled with the blue latex particles were then trapped by the rinderpest antibody in the result window, leading to an accumulation of the trapped complexes and thus the formation of a blue line. Since latex-bound antibody was applied in excess to the pad, unbound latex–antibody complexes travelled further to be trapped by a second anti-mouse antibody acting as an internal control. Interpretation of the test was simple, with two blue lines indicating RPV infection and one line indicating a RPV-negative result. The test was developed and commercialized by IAH Pirbright in collaboration first with Unipath and then Svanova Biotech (Brüning *et al.*, 1999; Brüning-Richardson *et al.*, 2011a). It was successfully used for the detection of rinderpest in Pakistan (Hussain *et al.*, 2001), and also used to detect RPV infections in Africa (Wambura *et al.*, 2000). The test compared favourably to the antigen-capture ELISA in terms of sensitivity. The simplicity of this test made it very attractive for use in the field and the format was successfully adapted for the detection of FMDV (Reid *et al.*, 2001) and also PPRV (Brüning-Richardson *et al.*, 2011a), which may be the next target for a global eradication programme (Anderson *et al.*, 2011). In addition, Brüning-Richardson *et al.* (2011b) recently reported that trapped rinderpest antigen contained within the devices was stable enough for confirmation by reverse transcription PCR (RT-PCR) in the laboratory.

6.5.3 Genome detection

Rinderpest-specific cDNA probes

New molecular technologies emerged during the 1980s, shifting the focus from the detection of viral nucleic acid rather than antigen. The RPV-specific cDNA probe technique allowed the hybridization of a single-stranded nucleic acid (the probe) to its complementary strand potentially present within the suspect sample. The reaction was detected by the addition of a label to the probe, which was either radioactive or non-radioactive. For the rinderpest-specific assay, Diallo *et al.* (1989) used cDNA clones corresponding to the RPV N-gene. The assay was further optimized to avoid the use of a radioactive label, because of the associated health hazards, by the incorporation of a biotin or digoxigenin label. However, even after the adoption of non-radioactive probes, results for this technique were disappointing with regards to sensitivity and the need for pure target RNA (Diallo *et al.*, 1989, 1995).

Reverse transcription-polymerase chain reaction (RT-PCR)

The 1980s brought a great revolution with regards to the molecular detection of infectious diseases. PCR amplification allowed the detection of even low levels of infectious agents by amplification from even low copy numbers of DNA. For RPV, the presence of an RNA genome meant that cDNA had to be generated first from a suspect sample because the RPV contained a single strand of negative-sense RNA. To do so, RNA extracted from suspect samples with phenol-chloroform was subjected to reverse-transcription (RT) generating DNA, which then was amplified using the specific rinderpest primers. Forsyth and Barrett (1995) were the first to develop a rinderpest-specific RT-PCR assay. This was based on the amplification of a specific segment of rinderpest DNA by use of the heat-resistant taq polymerase and rinderpest-specific primers, where double-stranded DNA was denatured by incubation at high temperatures, then allowed to be bound to the specific-sense and anti-sense rinderpest primers and amplified to generate new copies with the help of the taq polymerase. The newly created DNA was subjected to repeated cycles of denaturation, primer annealing and amplification, leading to the creation of a DNA product, which was visualized on an agarose gel after staining with ethidium bromide. The product size was

dependent on the specific primers used and was identified by the inclusion of a reference base pair ladder.

The first primer set used by Forsyth and Barrett (1995) targeted the phosphoprotein (P) gene, which is highly conserved among all morbilliviruses, followed by a rinderpest-specific primer set, which identified the virus fusion (F) gene. The advantage of this was to first determine if the suspect causative agent belonged to the morbilliviruses and then identify the virus species, since the second primer set detected a gene conserved among all rinderpest strains. In addition, a nested set of primers detecting a sequence within the amplified F gene was developed for confirmation of a negative result. Apart from its sensitivity for diagnostic purposes, the rinderpest RT-PCR also generated data for epidemiological studies. This was achieved by the sequencing of the PCR products and comparing the sequences with known RPV sequences held within a database. Rinderpest outbreaks could thus be confirmed and furthermore traced to their origin. Phylogenetic studies were now available that proved vital for the success of GREP and enabled the differentiation of RPV isolates into three distinct lineages (Fig. 6.4). Since the first RT-PCR for rinderpest was described, more assays have been developed. For example, Couacy-Hyman *et al.* (2006) described the development of a RT-PCR based on the amplification of the N gene. RNA extraction was simplified by the use of a commercial RNA extraction kit instead of phenol-chloroform-based extraction. Confirmation of results was strengthened after Southern blot transfer of the PCR product and probing with an internal SB1 probe, as well as cleavage of the PCR products with the restriction enzyme RsaI to give a RPV-specific band.

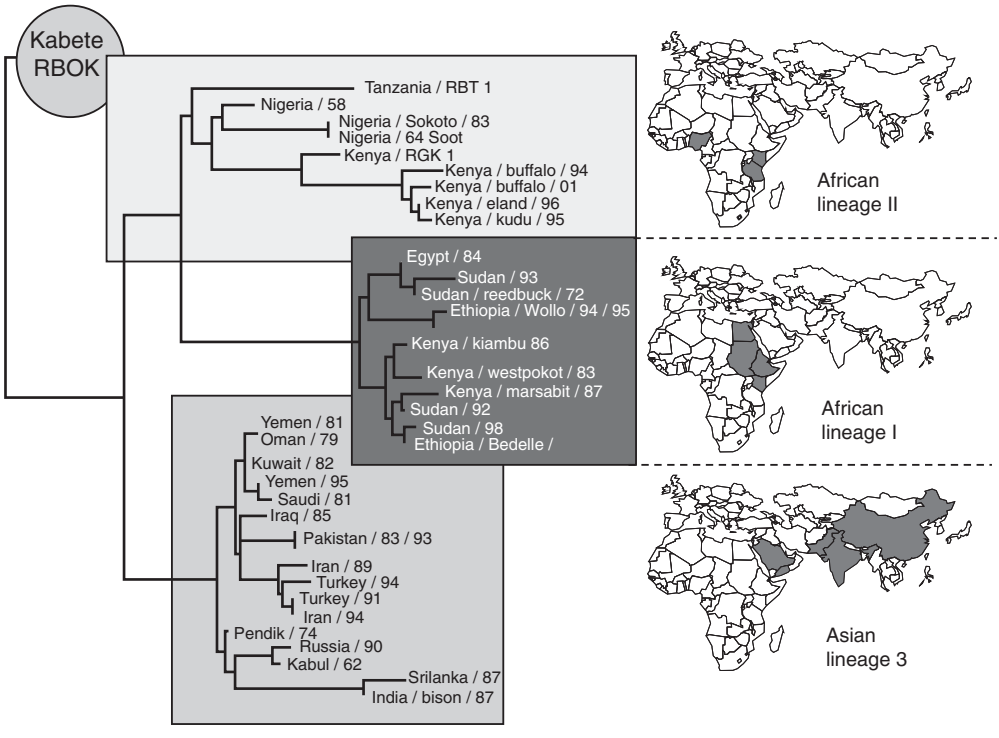

Fig. 6.4. Phylogenetic analysis of rinderpest isolates based of the F gene amplicon (Forsyth and Barrett, 1995). Lineages are detailed, as are shaded maps for each lineage illustrating the distribution of isolates from each lineage.

Molecular detection methods, including PCR technologies, continuously improved and gained acceptance as standard methods for RPV detection in developing countries where rinderpest remained a problem. Drawbacks to RT-PCR technologies included the need for specialized equipment and the need for highly skilled staff in resource-limited settings. However, the network of laboratories established during eradication campaigns ensured that samples could be transferred to national or world reference laboratories if methodologies were unavailable locally.

Real-time reverse transcription-PCR (rRT-PCR)

Most recently, an rRT-PCR for the specific detection of rinderpest has been described (Carrillo *et al.*, 2010). This assay was based on the detection of the L gene of the virus and was designed to allow a one-step detection of RPV with no cross reactivity with other closely related viruses such as PPRV or causative agents of diseases with similar clinical signs, including FMDV, vesicular stomatis virus, bluetongue virus and bovine herpes virus. The improvement of this test over conventional PCR methodologies was the enhanced sensitivity and specificity as well as the capability of a preclinical detection of the virus. This method generated rapid results in a portable format. It compared favourably to the virus isolation test with the PCR detecting RPV 1 or 2 days before virus isolation. As such it may be useful for the large-scale surveillance to monitor RPV re-emergence (Carrillo *et al.*, 2010). Apart from virus isolation and the techniques described here, other technologies have been developed, whose detailed description is beyond the scope of this chapter.

6.5.4 Antibody detection

Virus neutralization

The virus neutralization test was based on the interaction of antibodies with RPV leading to the neutralization of the virus particles, which rendered them non-infectious to cell culture. Test sera were taken from a suspect animal and serial dilutions were made. These dilutions were mixed with 100$TCID_{50}$ of live virus (usually the cell culture adapted 'Rinderpest Bovine Origin Kabete' (RBOK) virus vaccine). The virus/serum mixtures were added to bovine kidney or Vero cells and allowed to incubate for 14 days. The cells were inspected for CPE on days 3, 4, 7, 10, 12 and 14 post infection and the virus dose and virus neutralisation antibody titres were determined using negative and positive controls and a 50% end-point method. This test had several drawbacks, including the use of a live virus as well as the need for tissue culture facilities. In addition, the test took up to 2 weeks to confirm the presence of antibodies (Anderson *et al.*, 1996). The test was eventually replaced by the indirect ELISA and subsequently the competitive ELISA, which allowed rapid detection of antibodies (within 3 hours) using a standardized format.

Enzyme-linked immunosorbent assay (ELISA) for antibody detection

Indirect detection of rinderpest infection relied on the detection of antibody to rinderpest in the serum from infected animals. This method of detection was crucial for the initial phases of GREP, when it was important to monitor herd immunity among animals that had been vaccinated against rinderpest. As mentioned previously, a highly effective vaccine (Plowright/RBOK), which induced life-long immunity, had been developed, eliciting a high-titre antibody response among animals. This fact was exploited for the development of an indirect ELISA for the detection of circulating antibodies. The principle of the ELISA is the trapping of antibody by an immobilized antigen within a well of a 96-well plastic plate. The antigen–antibody interaction is detected by the addition of a secondary antibody conjugated with HRP and then taking colorimetric readings

on a special ELISA plate reader. Through the inclusion of positive and negative controls as well as the known limits for a positive result, it is possible to determine the presence of antibodies in a serum sample (Anderson *et al.*, 1982, 1983). However, the first ELISA format showed some drawbacks, including the need for species-specific secondary antibodies, which proved problematic when sera from wildlife were tested. Anderson and McKay (1994) improved the ELISA format by using mAbs specific for the RPV H-protein. In this improved assay, rinderpest antigen was used to coat 96 well plates. After incubation the plates were then incubated with the test serum. A new step was added by the addition of anti-rinderpest mAbs, which were allowed to compete with any antibody in the test serum for binding to the antigen. After an incubation period, a secondary anti-mouse antibody (which reacted with the mouse-derived mAb) with HRP-conjugate was then allowed to react. If no antibody was present, all of the mAbs were able to bind to the antigen resulting in colour formation. However, if antibodies to RPV were present in the sample less colour or no colour was visualized, since the antibodies in the test serum competed with the test antibody for antigen binding. Therefore, this ELISA was called a competitive ELISA because antibodies within suspect sera competed with the mAb for antigen binding. Again, the inclusion of negative and positive controls in the test allowed the calculation of inhibition values and therefore the detection of an antibody-positive sample. This test was an improvement on the earlier version by reducing the need for several secondary antibodies to just one and increasing specificity by the incorporation of a rinderpest-specific antibody. In addition, it allowed the standardization of the test to be used in GREP by supplying reference reagents (antigen, positive and negative controls) and internal quality-control values as well as a computer program for data analysis. The format proved to be so successful that IAH designed courses for the proper use of the assay, and supplied kits for use in GREP worldwide. Recent developments for competitive ELISAs include the cELISA based on mAbs to the H-protein of RPV (Singh *et al.*, 2000; Khamehchian *et al.*, 2007), mAbs to the secreted form of the H protein in a baculovirus expression system (Renukaradhya *et al.*, 2003) and a recombinant N protein and N protein mAbs-based assay (Choi *et al.*, 2003).

Advantages and disadvantages of the different assays mentioned in this chapter are summarized in Table 6.1. An exhaustive description of methods can be found in Anderson *et al.* (1996) and Taylor *et al.* (2005).

6.6 Concluding Remarks

Several factors contributed to the successful global rinderpest eradication campaign:

1. The existence of only one serotype of rinderpest virus (unlike, for example, foot and mouth disease virus, which has seven serotypes).
2. The availability of a highly effective attenuated vaccine that induces life-long immunity.
3. The development of sensitive and specific assays for large-scale sero-monitoring and virus detection.
4. Adoption of a single, quality-controlled assay for sero-monitoring and sero-surveillance with full technology transfer and technical back-stopping.
5. The evolution of participatory approaches in areas of civil unrest to deliver both sero-monitoring and disease surveillance.

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Table 6.1. Summary of the most commonly used techniques for RPV direct detection and serology.

Antigen detection	Sensitivity	Specificity	Sample	Comments	Reference
Virus isolation	NDA	RPV specific, but some reactivity with PPRV	Tissue	Result within 2 weeks; laboratory set-up required	Anderson *et al.* (1996)
Immunoperoxidase/ immuno fluorescence	NDA	Non-specific background; PPRV cross-reactivity	Tissue; LN	Result within 1–3 hours; slides can be stored	Anderson *et al.* (1996); Rossiter and Jessett (1982)
AGID	NDA	Some PPRV cross-reactivity	Ocular swab, gum debris; LN	Result within 12–36 hours; kit format	Forman *et al.* (1983); Obi and Patrick (1984)
CIEP	NDA	Some PPRV cross-reactivity	Ocular swab, gum debris; LN	Result within 40 minutes; adapted for field use	Rossiter and Mushi (1980); Injairu (1984)
IC-ELISA	$10^{2.2}TCID_{50}$/well	RPV specific	Tissue; LN; secretions	Result within 3 hours; large-scale testing; available in kit format	Libeau *et al.* (1994)
Genome detection					
RT-PCR rRT-PCR	0.59–87.5 $TCID_{50}$/2.5 µl reaction mix	RPV specific	Tissue; uncoagulated blood	Result within 5–6 hours; requires laboratory set-up; new version field adapted	Forsyth and Barrett (1995); Carrillo *et al.* (2010)
Chromatographic strip test	$10^{3.1–5.3}$ $TCID_{50}$/ml 10^{3} $TCID_{50}$/ml	RPV specific Some PPRV cross-reactivity	Ocular, nasal swab	Result within 20 minutes; user-friendly; kit format; for field use	Brüning *et al.* (1999); Brüning-Richardson *et al.* (2011a, 2011b)
Antibody detection					
Virus neutralization	NDA	RPV specific	Serum	Result within 2 weeks; requires laboratory set-up	Libeau *et al.* (1995); Anderson *et al.* (1996)
Competitive ELISA	70%	99%	Serum	Result within 3 hours; standardized; in kit format; large-scale testing	Anderson and McKay (1994)

NDA = no data available; AGID = agar gel immunodiffusion test; CIEP = counter-immunoelectrophoresis; IC-ELISA = immunocapture ELISA; RT-PCR = reverse-transcription PCR; rRT-PCR = real-time reverse transcription PCR; LN = lymph node

References

Ali, B., El, H. and Lees, G.E. (1979) The application of immunoelectroprecipitation in the diagnosis of rinderpest. *Bulletin of Animal Health and Production in Africa* 27, 1–6.

Anderson, J. and McKay, J.A. (1994) The detection of antibodies against peste des petits ruminants virus in cattle, sheep and goats and the possible implications to rinderpest control programmes. *Epidemiology & Infection* 112, 225–231.

Anderson, J., Rowe, L.W., Taylor, W.P. and Crowther, J.R. (1982) An enzyme-linked immunosorbent assay for the detection of IgG, IgA and IgM antibodies to rinderpest virus in experimentally infected cattle. *Research in Veterinary Science* 32, 242–247.

Anderson, J., Rowe, L.W. and Taylor, W.P. (1983) Use of an enzyme-linked immunosorbent assay for the detection of IgG antibodies to rinderpest virus in epidemiological surveys. *Research in Veterinary Science* 34, 77–81.

Anderson, J., Barrett, T. and Scott, G.R. (1996) Immunocapture ELISA. In: *Manual on the Diagnosis of Rinderpest*, Chapter 7. FAO Animal Health Manual, FAO, Rome, pp. 63–67.

Anderson, J., Baron, M., Cameron, A., Kock, R., Jones, B., Pfeiffer, D., Mariner, J., McKeever, D., Oura, C., Roeder, P., Rossiter, P. and Taylor, W. (2011) Rinderpest eradicated; what next? *Veterinary Record* 169, 10–11.

Baron, M.D. and Barrett, T. (2000) Rinderpest virus lacking the C and V proteins show specific defects in growth and transcription of viral RNAs. *Journal of Virology* 74, 2603–2611.

Baron, M.D., Goatley, L. and Barrett, T. (1994) Cloning and sequence analysis of the matrix (M) protein gene of rinderpest virus and evidence for another bovine morbillivirus. *Virology* 200, 121–129.

Bellini, W.J., Englund, G., Rozenblatt, S., Arnheiter, H. and Richardson, C.D. (1985) Measles virus P gene codes for two proteins. *Journal of Virology* 53, 908–919.

Blixenkrone-Møller, M., Bolt, G., Jensen, T.D., Harder, T. and Svansson, V. (1996) Comparative analysis of the attachment protein gene (H) of dolphin morbillivirus. *Virus Research* 40, 47–55.

Bolt, G., Jensen, T.D., Gottschalck, E., Arctander, P., Appel, M.J., Buckland, R. and Blixenkrone-Møller, M. (1997) Genetic diversity of the attachment (H) protein gene of current field isolates of canine distemper virus. *Journal of General Virology* 78, 367–372.

Brown, C.C. (1999) A review of three pathology-based techniques for retrospective diagnosis of rinderpest, with comparison to virus isolation. *Research in Veterinary Science* 63, 103–106.

Brown, C.C. and Torres, A. (1994) Distribution of antigen in cattle infected with rinderpest virus. *Veterinary Pathology* 31, 194–200.

Brown, C.C., Ojok, L. and Mariner, J.C. (1996) Immunohistochemical detection of rinderpest virus: effects of autolysis and period of fixation. *Research in Veterinary Science* 60, 182–184.

Brown, D.D., Rima, B.K., Allen, I.V., Baron, M.D., Banyard, A.C., Barrett, T. and Duprex, W.P. (2005) Rational attenuation of a morbillivirus by modulating the activity of the RNA-dependent RNA polymerase. *Journal of Virology* 79, 14,330–14,338.

Brüning, A., Bellamy, K., Talbot, D. and Anderson, J. (1999) A rapid chromatographic strip test for the pen-side diagnosis of rinderpest virus. *Journal of Virological Methods* 81, 143–154.

Brüning-Richardson, A., Barrett, T., Garratt, J.C. and Anderson, J. (2011a) The detection of rinderpest virus RNA extracted from a rapid chromatographic strip-test by RT-PCR. *Journal of Virological Methods* 173, 394–398.

Brüning-Richardson, A., Akerblom, L., Klingeborn, B. and Anderson, J. (2011b) Improvement and development of rapid chromatographic strip-tests for the diagnosis of rinderpest and peste des petits ruminants viruses. *Journal of Virological Methods* 174, 42–46.

Buczkowski, H., Parida, S., Bailey, D., Barrett, T. and Banyard, A.C. (2012) A novel approach to generating morbillivirus vaccines: negatively marking the rinderpest vaccine. *Vaccine* 30, 1927–1935.

Carrillo, C., Prarat, M., Vagnozzi, A., Calahan, J.D., Smoliga, G., Nelson, W.M. and Rodriguez, L.L. (2010) Specific detection of Rinderpest virus by real-time reverse transcription-PCR in preclinical and clinical samples from experimentally infected cattle. *Journal of Clinical Microbiology* 48, 4094–4101.

Choi, K.S., Nah, J.J., Choi, C.U., Ko, Y.J., Sohn, H.J., Libeau, G., Kang, S.Y. and Joo, Y.S. (2003) Monoclonal antibody-based competitive ELISA for simultaneous detection of rinderpest virus and peste des petits ruminants virus antibodies. *Veterinary Microbiology* 96, 1–16.

Couacy-Hymann, E., Bodjo, S.C., Danho, T., Koffi, M.Y. and Akoua-Koffi, C. (2006) Diagnosis and surveillance of rinderpest using reverse transcription-PCR. *African Journal of Biotechnology* 5, 1717–1721.

Curran, J. and Kolakofsky, D. (1999) Replication of paramyxoviruses. *Advances in Virus Research* 54, 403–422.

Curran, M.D., Lü, Y.J. and Rima, B.K. (1992) The fusion protein gene of phocine distemper virus: nucleotide and deduced amino acid sequences and a comparison of morbillivirus fusion peptides. *Archives of Virology* 126, 159–169.

Das, S.C., Baron, M.D. and Barrett, T. (2000) Recovery and characterization of a chimeric rinderpest virus with the glycoproteins of peste-des-petits-ruminants virus: homologous F and H proteins are required for virus viability. *Journal of Virology* 74, 9039–9047.

Diallo, A., Barrett, T., Barbron, M., Subbarao, S.M. and Taylor, W.P. (1989) Differentiation of rinderpest and peste des petits ruminants viruses using specific cDNA clones. *Journal of Virological Methods* 23, 127–136.

Diallo, A., Libeau, G., Couacy-Hymann, E. and Barbron, M. (1995) Recent developments in the diagnosis of rinderpest and peste des petits ruminants. *Veterinary Microbiology* 44, 307–317.

Duprex, W.P., Collins, F.M. and Rima, B.K. (2002) Modulating the function of the measles virus RNA-dependent RNA polymerase by insertion of green fluorescent protein into the open reading frame. *Journal of Virology* 76, 7322–7328.

Escoffier, C., Manié, S., Vincent, S., Muller, C.P., Billeter, M. and Gerlier, D. (1999) Nonstructural C protein is required for efficient measles virus replication in human peripheral blood cells. *Journal of Virology* 73, 1695–1698.

FAO (2002) EMPRES Transboundary Animal Diseases Bulletin 20/1–2002 (ftp://ftp.fao.org/docrep/fao/004/y3649e/y3649e00.pdf).

FAO (2011) Rinderpest eradicated – what next? (http://www.fao.org/news/story/en/item/80894/icode/ accessed 22 April 2013).

Forman, A.J., Rowe, L.W. and Taylor, W.P. (1983) Detection of rinderpest antigen by agar gel diffusion and counter-immunoelectrophoresis. *Tropical Animal Health and Production* 15, 83–85.

Forsyth, M.A. and Barrett, T. (1995) Evaluation of polymerase chain reaction for the detection and characterisation of rinderpest and peste des petits ruminants viruses for epidemiological studies. *Virus Research* 39, 151–163.

Gerlier, D. and Lyles, D.S. (2011) Interplay between innate immunity and negative-strand RNA viruses: towards a rational model. *Microbiology and Molecular Biology Reviews* 75, 468–490.

Horikami, S.M., Curran, J., Kolakofsky, D. and Moyer, S.A. (1992) Complexes of Sendai virus NP-P and P-L proteins are required for defective interfering particle genome replication in vitro. *Journal of Virology* 66, 4901–4908.

Hussain, M., Iqbal, M., Taylor, W.P. and Roeder, P.L. (2001) Pen-side test for the diagnosis of rinderpest in Pakistan. *Veterinary Record* 149, 300–302.

Injairu, R.M. (1984) CIEP in the field. Dissertation, University of Edinburgh, UK.

Kato, A., Kiyotani, K., Sakai, Y., Yoshida, T. and Nagai, Y. (1997) The paramyxovirus, Sendai virus, V protein encodes a luxury function required for viral pathogenesis. *EMBO Journal* 16, 578–587.

Khamehchian, S., Madani, R., Rasaee, M.J., Golchinfar, F. and Kargar, R. (2007) Development of 2 types of competitive enzyme-linked immunosorbent assay for detecting antibodies to the rinderpest virus using a monoclonal antibody for a specific region of the hemagglutinin protein. *Canadian Journal of Microbiology* 53, 720–726.

Lamb, R.A. and Kolakofsky, D. (2001) Paramyxoviridae: the viruses and their replication. In: Knipe, D.M. and Howley, P.M. (eds) *Fields Virology*. Lippincott Williams & Wilkins, Philadelphia, Pennsylvania, pp. 1305–1340.

Langedijk, J.P., Daus, F.J. and van Oirschot, J.T. (1997) Sequence and structure alignment of Paramyxoviridae attachment proteins and discovery of enzymatic activity for a morbillivirus hemagglutinin. *Journal of Virology* 71, 6155–6167.

Libeau, G., Diallo, A., Calvez, D. and Lefèvre, P.C. (1992) A competitive ELISA using anti-N monoclonal antibodies for specific detection of rinderpest antibodies in cattle and small ruminants. *Veterinary Microbiology* 31, 147–160.

Libeau, G., Diallo, A., Colas, F. and Guerre, L. (1994) Rapid differential diagnosis of rinderpest and peste des petits ruminants using an immunocapture ELISA. *Veterinary Record* 134, 300–304.

Libeau, G., Préhaud, C., Lancelot, R., Colas, F., Guerre, L., Bishop, D.H. and Diallo, A. (1995) Development of a competitive ELISA for detecting antibodies to the peste des petits ruminants virus using a recombinant nucleoprotein. *Research in Veterinary Science* 58, 50–55.

Limo, M. and Yilma, T. (1990) Molecular cloning of the rinderpest virus matrix protein gene: comparative sequence analysis with other paramyxoviruses. *Virology* 175, 323–327.

Mahapatra, M., Parida, S., Egziabher, B.G., Diallo, A. and Barrett, T. (2003) Sequence analysis of the phosphoprotein gene of peste des petits ruminants (PPR) virus: editing of the gene transcript. *Virus Research* 96, 85–98.

Mahapatra, M., Parida, S., Baron, M.D. and Barrett, T. (2006) Matrix protein and glycoproteins F and H of Peste-des-petits-ruminants virus function better as a homologous complex. *Journal of General Virology* 87, 2021–2029.

Mahy, B.W.J., Barrett, T. Evans, S., Anderson, E.C. and Bostock, C.J. (1988) Characterization of a seal morbillivirus. *Nature* 336, 115–116.

Massé, N., Ainouze, M., Néel, B., Wild, T.F., Buckland, R. and Langedijk, J.P. (2004) Measles virus (MV) hemagglutinin: evidence that attachment sites for MV receptors SLAM and CD46 overlap on the globular head. *Journal of Virology* 78, 9051–9063.

Obi, T.U. and Patrick, D. (1984) The detection of peste des petits ruminants (PPR) virus antigen by agar gel precipitation test and counter-immunoelectrophoresis. *Journal of Hygiene* 93, 579–586.

Reid, S.M., Ferris, N.P., Brüning, A., Hutchings, G.H., Kowalska, Z. and Akerblom, L. (2001) Development of a rapid chromatographic strip test for the pen-side detection of foot-and-mouth disease virus antigen. *Journal of Virological Methods* 96, 189–202.

Renukaraddhya, G.J., Suresh, K.B., Rajasekhar, M. and Shaila, M.S. (2003) Competitive enzyme-linked immunosorbent assay based on monoclonal antibody and recombinant haemagglutinin for serosurveillance of rinderpest virus. *Journal of Clinical Microbiology* 41, 943–947.

Roeder, P. (2011a) Making a global impact: the eradication of rinderpest. *Veterinary Record* 169, 650–652.

Roeder, P. (2011b) A world without rinderpest. *Veterinary Record* 168, 96–97.

Rossiter, P.B. (1984) Notes on immune-precipitin reactions with rinderpest virus. *Tropical Animal Health and Production* 17, 55–56.

Rossiter, P.B. and Jessett, D.M. (1982) Detection of rinderpest virus antigens in vitro and in vivo by direct immunofluorescence. *Research in Veterinary Science* 33, 198–204.

Rossiter, P.B. and Mushi, E.Z. (1980) Rapid detection of rinderpest virus antigen by counter-immunoelectrophoresis. *Tropical Animal Health and Production* 12, 209–216.

Sedlmeier, R. and Neubert, W.J. (1998) The replicative complex of paramyxoviruses: structure and function. *Advances in Virus Research* 50, 101–139.

Shah, R.A., Joseph, M.C., Butchaiah, G., Malik, M., Singh, R.K. and Bakshi, C.S. (2004) Detection of rinderpest virus using N-protein monoclonal antibodies. *Tropical Animal Health and Production* 36, 11–25.

Sheshberadaran, H., Norrby, E., McCullough, K.C., Carpenter, W.C. and Orvell, C. (1986) The antigenic relationship between measles, canine-distemper and rinderpest viruses studied with monoclonal-antibodies. *Journal of General Virology* 67, 1381–1392.

Singh, R.P., Sreenivasa, B.P., Dhar, P., Roy, R.N. and Bandyopadhyay, S.K. (2000) Development and evaluation of a monoclonal antibody based competitive enzyme-linked immunosorbent assay for the detection of rinderpest virus antibodies. *Revue Scientifique et Technique (International Office of Epizootics)* 19, 754–763.

Stricker, R., Mottet, G. and Roux, L. (1994) The Sendai virus matrix protein appears to be recruited in the cytoplasm by the viral nucleocapsid to function in viral assembly and budding. *Journal of General Virology* 75, 1031–1042.

Sweetman, D.A., Miskin, J. and Baron, M.D. (2001) Rinderpest virus C and V proteins interact with the major (L) component of the viral polymerase. *Virology* 281, 193–204.

Taylor, W.P., Barrett, T. and Pastoret, P.-P. (eds) (2005) *Rinderpest and Peste des Petits Ruminants: Virus Plagues of Large and Small Ruminants*. Elsevier, Academic Press, London.

Wambura, P.N., Moshy, D.W., Mbise, A.N., Mollel, G.O., Taylor, W.P., Anderson, J. and Bruning, A. (2000) Diagnosis of rinderpest in Tanzania by a rapid chromatographic strip-test. *Tropical Animal Health and Production* 32, 141–145.

Wild, T.F., Naniche, D., Rabourdin-Combe, C., Gerlier, D., Malvoisin, E., Lecouturier, V. and Buckland, R. (1995) Mode of entry of morbilliviruses. *Veterinary Microbiology* 44, 267–270.

7 Bovine Parainfluenza Virus 3

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7.1 History and Importance

Bovine parainfluenza virus 3 (BPIV-3) was first isolated in the USA from the nasal discharge of calves showing clinical signs of shipping fever, including rapid onset of fever, nasal discharge, coughing and other respiratory signs, lacrimation, conjunctivitis and lack of appetite (Andrewes *et al.*, 1959; Reisinger *et al.*, 1959). Since then, the virus has been reported globally from infected and clinically normal cattle. It is generally accepted that co-infection of BPIV-3 along with other viruses and *Mannheimia*/*Pasturella* species could result in a clinical disease known as shipping fever, now known as undifferentiated fever/bovine respiratory disease complex (UF/BRDC) (Edwards, 2010). A variety of factors, such as environmental temperature, transportation, hygiene, stocking density, co-mingling and host immune status, can contribute to increased susceptibility to secondary bacterial infection and severity of clinical disease. UF/BRDC is the most important cause of economic losses for the stocker and feedlot production systems (Gagea *et al.*, 2006; Snowder *et al.*, 2006) in many countries where the climate provides an additional stress. Poor growth performance, therapeutic costs, mortality and reduced carcass value contribute to these losses, estimated to be about US$1 billion per year (Griffin, 1997). Research on the viral aetiology of UF/BRDC has largely focused on bovine herpes virus-1 (BHV-1), bovine viral diarrhoea virus (BVDV) and bovine respiratory syncytial virus (BRSV). Few publications have considered HPIV-3 on its own, with most reports including BPIV-3 as one among multiple viruses and bacteria described (Fulton, 2009). The role of BPIV-3 in UF/BRDC is still elusive because of its endemicity, concurrent infections with other viruses, bacteria and *Mycoplasma*, and the continued use of modified live virus vaccines.

7.2 Epizootiology

BPIV-3 has a worldwide distribution. BPIV-3 infections were found in a variety of species including cattle (Andrewes *et al.*, 1959; Reisinger *et al.*, 1959), humans, sheep (Lyon *et al.*, 1997), goats (Yener *et al.*, 2005), bison (Zarnke and Erickson, 1990), water buffalo (Maidana *et al.*, 2012), guinea pigs (Ohsawa *et al.*, 1998), black and white rhinoceros (Fischer-Tenhagen *et al.*, 2000), moose (Thorsen and Henderson, 1971), bighorn sheep (Parks *et al.*, 1972) and camels (Eisa *et al.*, 1979). Cross-species infection has been reported in humans, sheep

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and non-human primates with bovine PIV-3 (Stevenson and Hore, 1970). Unlike HPIV-3, human-to-human transmission of bPIV-3 is poor. Between susceptible animals, the virus is transmitted by aerosol and fomites contaminated with nasal discharges. The infection is generally subclinical in calves, kids and lambs, but may manifest as severe respiratory disease and pneumonia. The role of BPIV-3 in shipping fever is fiercely debated. However, poor hygiene, crowding, transport, harsh climatic conditions and other stressors typically result in UF/BRDC where BPIV-3 may or may not be involved.

7.3 Infectious Agent

7.3.1 Classification

BPIV-3 is an enveloped, non-segmented, negative-sense, single-stranded RNA virus classified in the genus *Respirovirus* in the *Paramyxoviridae* family, order *Mononegavirales* (King *et al*., 2012). The other members of the genus *Respirovirus* include human parainfluenza viruses 1 and 3 (HPIV1 and HPIV3), and Sendai virus (Karron and Collins, 2007), Interestingly, three of the five genera of the subfamily *Paramyxovirinae* include parainfluenza viruses (PIV), which share physical and morphologic properties (Karron and Collins, 2007). Genus *Rubulavirus* contains HPIV2, SV5, SV41 and mumps viruses, while genus *Avulavirus* contains avian paramyxoviruses (APMV) serotypes 1 through 9, of which Newcastle disease virus is the prototype APMV-1 (Karron and Collins, 2007). The virions of PIV are morphologically indistinguishable and also share distinct biophysical characteristics. All of them possess haemagglutinating and neuraminidase activities (Karron and Collins, 2007). There are three genotypes of BPIV-3 designated A, B and C. Previously, the complete genome analyses of the representative BPIV-3 isolates from Australia and North America indicated that BPIV-3 falls into two distinct genotypes, BPIV-3 genotype A (BPIV-3a) and BPIV-3 genotype B (BPIV-3b) (Horwood *et al*., 2008). Recently, four isolates, which were only detected in China, were proposed as genotype C, (Zhu *et al*., 2011).

7.3.2 Virion structure

The family *Paramyxoviridae* has a typical negative-stranded RNA virus structural pattern. The enveloped virus particle contains a left-handed, helically symmetrical ribonucleoprotein core, called the nucleocapsid, which consists of a non-segmented single-negative-stranded viral RNA genome surrounded by nucleocapsid proteins and associated with phosphoprotein and a large polymerase protein (Lamb and Parks, 2007). The nucleocapsid of *Paramyxovirinae*, 18 nm in diameter, 1 μm in length and a pitch of 5.5 nm in diameter, is enclosed by an outer lipoprotein envelope (Lamb and Parks, 2007). The helical nucleocapsid of paramyxoviruses presents a 'herring bone' morphology when viewed under an electron microscope. *Pneumovirinae* subfamily can be differentiated from *Paramyxovirinae* subfamily morphologically because they have narrower nucleocapsids (Lamb and Parks, 2007). The virions of *Paramyxoviridae* are pleomorphic in shape, being spherical or filamentous. In general, spherical virions are 150–350 nm in diameter (Lamb and Parks, 2007). Like other PIV, BPIV-3 virions are pleomorphic 150–300 nm in diameter. BPIV-3 has a buoyant density of 1.197. The RNA of BPIV-3, like that of Sendai virus, is a single continuous chain that lacks polyadenylic acid sequences and tends to self-anneal to a marked extent. It has a sedimentation coefficient of 42S and a molecular weight of 4.5×10^6 (Shibuta *et al*., 1979).

7.3.3 Genome organization

The genome length of family *Paramyxoviridae* ranges from 15,000 to 19,000 nucleotides (nt), encoding 6–10 tandem linked genes in the order 3′-N-P-M-F-HN-L-5, where N, P, M, F,

HN and L indicate the genes for the nucleocapsid protein, phosphoprotein, matrix, fusion, attachment and large polymerase proteins (Karron and Collins, 2007). The phosphoproteins encode additional accessory proteins by a process called 'RNA editing'. The genomic map of a typical parainfluenzavirus is shown (Fig. 7.1). Interestingly, the genome length of most members of *Paramyxoviridae* is divisible by six, known as the 'rule of six' (Calain and Roux, 1993; Kolakofsky *et al.*, 1998, 2005), i.e. their genomes must be of polyhexameric length (6n+0) to replicate efficiently. Each nucleoprotein subunit associates with 6 nt and that makes the RNA chain length an even multiple of six when completely encapsidated. There is a ~50 nt 3′ extracistronic leader region and a 50–171 nt 5′ extracistronic trailer region (or [–] leader) at the two ends of the genome. The leader and trailer are considered to be *cis*-acting signals and are essential for transcription and replication. Between the gene boundaries, there are intergenic regions, which are strictly conserved trinucleotides in *Respirovirus*, *Henipavirus* and *Morbillivirus*, but quite variable in length for the *Rubulavirus* and *Pneumovirinae*, ranging from 1 to 56 nt. The newly discovered paramyxoviruses, such as Tioman virus, have even longer intergenic regions, which may be up to 70 nt in length. Similar to other *Respiroviruses*, BPIV-3 consists of six nonoverlapping genes, which are predicted to encode nine proteins with conserved and complementary 3′ leader and 5′ trailer regions, conserved gene starts, gene stops and trinucleotide intergenic sequences. The genome of BPIV-3 follows the 'rule of six'.

7.4 Viral Proteins

Envelope glycoprotein complexes of *Paramyxoviridae* are stalk-like spikes on the surface of virions. Fusion protein (F) and an attachment protein, called haemagglutinin-neuraminidase (HN) or haemagglutinin (H) or glycoprotein (G) depending on the virus genera (Lamb and Parks, 2007), are the major components of the spikes. The F and HN/H/G proteins may be in the form of trimers (F) and tetramers (attachment protein), respectively. The envelope glycoproteins are responsible for mediating virus attachment and penetration during infection. The internal helical nucleocapsid core contains the RNA genome and nucleocapsid (N), phospho- (P) and large (L) polymerase

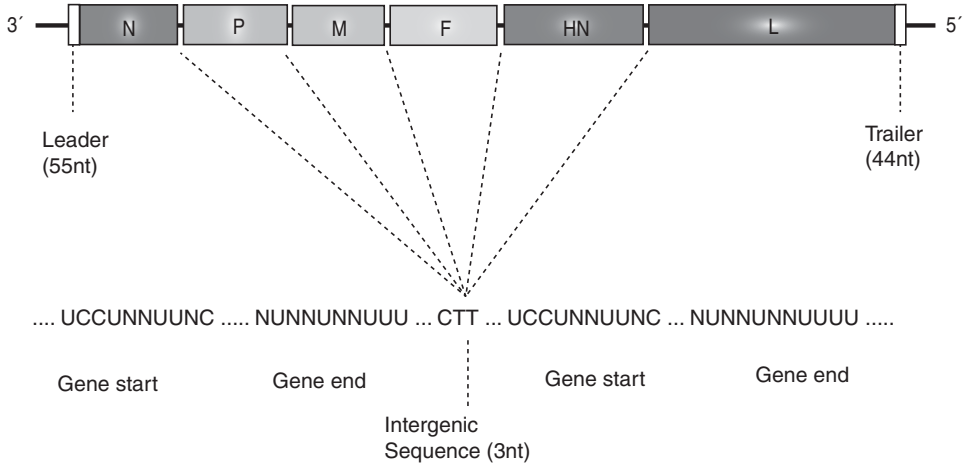


Fig. 7.1. Schematic of genome organization of BPIV-3 (not to scale). The BPIV-3 genome is depicted as a rectangle with vertical bars denoting the intergenic regions. The 3′ leader and the 5′ trailer regions are shown. The gene-end and gene-start sequences flanking each of the intergenic sequences are depicted. The P gene also encodes V/D/W and C proteins. The genome termini carry complementary sequences.

proteins. Another structural protein, matrix (M) protein, is located between the envelope and the core, and is important in virion architecture and the infection process. Accessory proteins are mostly generated from overlapping open reading frames within P gene transcriptional units by a process called RNA editing or by alternative translation initiation. These accessory proteins are important in viral morphogenesis, RNA synthesis and pathogenesis (Lamb and Parkes, 2007).

7.4.1 Nucleocapsid protein

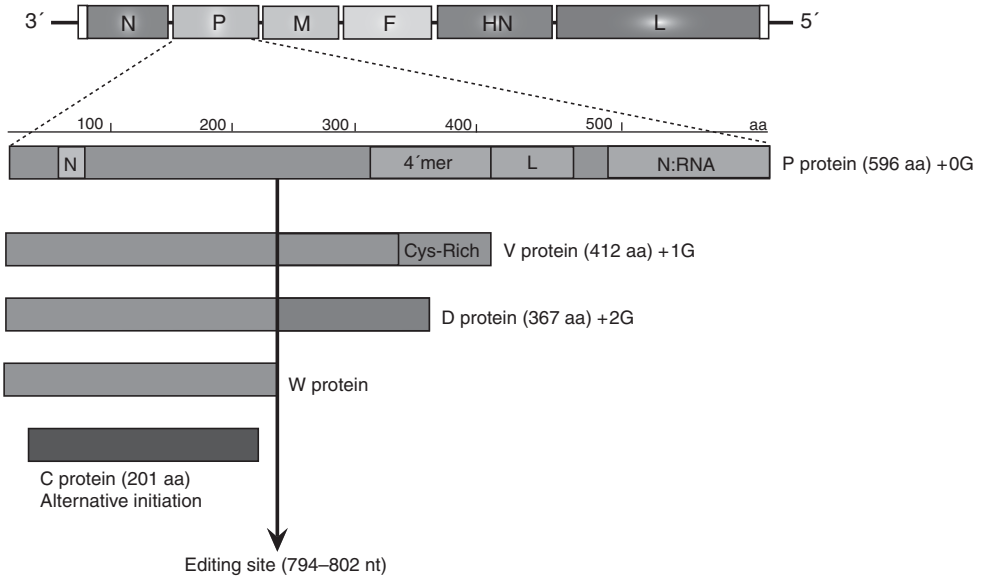
The N protein of BPIV-3 is one of the most abundant proteins and is present as the first transcribed gene, adjacent to the leader sequence at the 3′ end of viral genomic RNA. The N protein is composed of 489–553 amino acids (aa) with the predicted molecular weight of 53–58 kDa. The N-terminal region of the N protein of paramyxoviruses is involved with encapsidation of viral RNA, and coats the full length of viral [–] sense genomic and [+] sense antigenomic RNAs to form the helical nucleocapsid. This helical structure serves several functions, including protection from nuclease digestion, alignment of distal RNA segments to create a functional 3′-end promoter and providing interaction with P and L proteins during transcription and replication, and association with M protein during virus assembly (Lamb and Parks, 2007). Sendai virus N binds approximately six consecutive nt and 13 subunits constitute each turn of the nucleocapsid helix (Egelman *et al.*, 1989). But slight differences in the number of N subunits per helix turn and pitch of the helix have been reported among paramyxoviruses (Bhella *et al.*, 2002). BPIV-3 N is expected to bind the genome and antigenome similar to that of SeV. N protein exists in at least two forms in infected cells. The first form is tightly associated with RNA in a nucleocapsid structure (Karron and Collins, 2007). The second unassembled soluble form termed N^0 has been shown to be associated with P protein for a number of paramyxoviruses, including SeV (Horikami *et al.*, 1992), SV5 (Precious *et al.*, 1995), hPIV 3 (Zhao and Banerjee, 1995) and others (Karron and Collins, 2007).

7.4.2 The P gene and its encoded proteins

Paramyxovirinae P gene encoding multiple viral proteins can be considered to be a remarkable example of exploiting the coding capacity of viruses. SeV P gene can direct the expression of at least seven polypeptides, including P, V, W, C′, C, Y1 and Y2. Other paramyxoviruses may express fewer proteins from the P gene, but this capability is always seen. Generally, two mechanisms involve production of multiple mRNA species from the P gene. One is known as alternative initiation, by which the family of C proteins is produced from an alternative translation initiation codon. The second mechanism involves pseudotemplated insertion of multiple G residues known as 'RNA editing' at a specific position, termed the mRNA editing site, to produce mRNAs of P, V proteins and virus-specific proteins variously called W/D/I proteins, depending on the virus genus. P, V, W/D/I are a set of proteins that are amino co-terminal and the mRNAs are only different by inserted G nucleotides that shift the translation reading frame after the insertion site (Lamb and Parkes, 2007). In BPIV-3, the P protein is encoded as the translation product from the unedited mRNA (+0G), the V protein by a transcript containing a single G residue at the editing site (+1G) and the D protein by the insertion of two G nucleotides (+2G), but the C protein is produced by alternate translation initiation (Karron and Collins, 2007).

The P protein of PIV is essential for viral RNA synthesis and it interacts with multiple partners during the viral growth cycle (Karron and Collins, 2007). The paramyxovirus P protein is generally 400–600-aa long. It is heavily phosphorylated at serine and threonine residues predominantly within the N-terminal region (Lamb and Parks, 2007). Distinct modular carboxy and N-terminal domains within the P protein play essential roles as a polymerase

co-factor and in nascent chain assembly. The C-terminal polymerase co-factor module is conserved in the predicted secondary structure for all *Paramyxovirinae* members. It contains domains for P–P multimerization, for L protein interactions and for N–RNA template binding (Horikami *et al.*, 1992). The N-terminal region is believed to act as a chaperone to facilitate interactions with unassembled N in order to prevent N aggregation and to prevent uncontrolled encapsidation of non-viral RNA (Curran *et al.*, 1995). A defined N-terminal region of P up to residue 78 is required for genome replication and assembly as deletion of residues 78–324 still makes the protein active (Curran *et al.*, 1985; Karron and Collins, 2007). The P protein functions as a multimer and Sev P protein is a tetramer (Tarbouriech *et al.*, 2000a, 2000b). Corresponding domain structure and functional studies with BPIV-3 P proteins are lacking but a comparative domain structure with P-gene RNA editing strategy is shown (Fig. 7.2).



BPIV3	AAAAAA	GGG
Respirovirus	AAAAAA	GGG
Morbillivirus	AAAAA	GGG
Rubulavirus	AAGA	GGGG(n)
Avulavirus	AAAAA	GGG
Henipavirus	AAAAA	GGG
ASPV	AAAAAA	GG
J-V	AAAAAA	GG
BeV	AAAAA	GG
FDLV	AUAA	GGGGGG
TPMV	AAAAAA	GG

Fig. 7.2. Schematic and structure-based sequence alignment of BPIV-3 P/V/D and C proteins. The P, V and D proteins: rectangular boxes with a common NH_2 terminal domain but unique C-terminal domains that result from addition of 1 (V) or 2 non-templated G (D) residues. Vertical line: the RNA editing site from where the point of sequence divergence starts. The P gene mRNA editing site of various members of the *Paramyxovirinae* is compared. The free N^0 binding region of P protein is shown at the amino-terminal. The oligomerization domain of the P protein at the C-terminal is shown as 4′mer. The L–P interaction domain is shown as L. The extreme C-terminal domain of the P protein is required for the interaction of the P protein to the N-RNA template. The C protein is expressed from the P mRNA using a +1 reading frame with respect to the P open reading frame. BPIV-3: Bovine parainfluenzavirus 3, ASPV: Atlantic salmon paramyxovirus, J-V: J virus, BeV: Beilong virus, FDLV: Fer-de-Lance virus, TPMV: Tupaia paramyxovirus.

7.4.3 Viral accessory proteins and their interactions with the host

The type I interferon (IFN) response is one of the most important antiviral responses and has two general phases: a primary transcriptional phase of induction of IFN synthesis, and a secondary transcriptional phase through the type I IFN signaling pathway (Borden *et al.*, 2007). Paramyxovirus accessory proteins can counteract the host-cell IFN pathways at both levels. The paramyxovirus V protein cysteine-rich domain can limit dsRNA-induced activation of the IFN-β promoter by direct interaction with melanoma differentiation-associated gene 5 (MDA5) (Andrejeva *et al.*, 2004; Komatsu *et al.*, 2004). The expression of C proteins in SeV and W proteins in NiV can also reduce IFN-β promoter activation in response to dsRNA (Komatsu *et al.*, 2004). IFN signaling is initiated by binding of secreted IFN and its cognate receptor on the cell surface leading to phosphorylation of latent transcription factors, STAT1 and STAT2, which are signal transducers and activators of transcription (Karron and Collins, 2007; Lamb and Parks, 2007). The V protein of some paramyxoviruses such as PIV5, MuV, SV41, hPIV2 and NDV can target one of the STATs for degradation to block the IFN signaling (Goodbourn and Randall, 2009), while other V proteins, like those of henipaviruses and measles virus, can form a V and STAT protein binding complex and prevent its translocation to the nucleus (Palosaari *et al.*, 2003; Rodriguez *et al.*, 2003; Shaw *et al.*, 2004). The C protein of respiro- and morbilliviruses are involved in blocking IFN signaling by a different mechanism (Garcin *et al.*, 2001, 2002; Gotoh *et al.*, 2003). SeV and HPIV3 can alter STAT phosphorylation patterns (Gotoh *et al.*, 2003), interact with STAT1 (Garcin *et al.*, 2001; Takeuchi *et al.*, 2001), and induce ubiquitination and degradation of STAT1 in some mouse cells (Garcin *et al.*, 2002).

V protein

The V protein is a 25–30 kDa polypeptide that is amino co-terminal with P protein but with a variable C-terminal domain. The C-terminal V-specific domain is highly conserved among paramyxoviruses with invariantly spaced histidine and cysteine residues forming a domain (cys-rich domain) that binds two zinc molecules per V protein (Liston and Briedis, 1994; Paterson *et al.*, 1995; Fukuhara *et al.*, 2002). The V protein plays important roles in virus replication (Curran *et al.*, 1991; Baron and Barrett, 2000), functions as a negative regulator to inhibit RNA synthesis (Delenda *et al.*, 1997; Durbin *et al.*, 1999; Baron and Barrett, 2000) and inhibits host-cell antiviral response by interacting with cellular proteins (Lin *et al.*, 1998; Andrejeva *et al.*, 2002). The V protein of BPIV-3 has been shown to target melanoma differentiation antigen-5 (MDA5), but not RIG-I for blocking the IFN-β activation signal (Komatsu *et al.*, 2007). Interestingly, it does not suppress IFN-β production induced by TRIF, TBK1 and IKKi molecules downstream of MDA5 and RIG-I (Komatsu *et al.*, 2007).

W/D/I protein

The W and D proteins are expressed by inserting two G residues at the P gene mRNA editing site in respiro-, morbilli- and henipaviruses. The W protein in SeV is found to interact with unassembled β, suggesting an inhibitory role in viral RNA synthesis (Horikami *et al.*, 1996). In PIV3, insertion of two G residues at the editing site produces a protein called D protein (Galinski *et al.*, 1992). In *Rubulaviruses*, insertion of one or four G residues during RNA editing produces I protein (Thomas *et al.*, 1988; Paterson and Lamb, 1990). Despite high sequence homology (~59%) between the cysteine-rich carboxy terminus of V proteins of BPIV-3 and SeV, there is high sequence diversity in the carboxy terminal regions between the D protein of BPIV-3 and W protein of SeV (Nagai, 1999). The D protein consists of the P–V amino co-terminal region (241 aa) plus a long unique carboxy terminal region (Fig. 7.2), while the W open reading frame is terminated by a stop codon immediately after the editing site. The role of W/D/I in the viral growth cycle has not been understood (Lamb and

Parks, 2007). The D protein of BPIV-3 does not block type I IFN in infected cells (Komatsu *et al.*, 2007). The exact function of the D protein is unknown. I protein is not produced by BPIV-3. It is only seen in members of *Rubulavirus* due to the insertion of either one or four G residues to the P mRNA during RNA editing.

C protein

C proteins are generated using alternative translation initiation codons (+1 reading frame with respect to the P open reading frame) in *Respiro-*, *Henipa-*, *Morbillivirus* (Lamb and Parks, 2007). In SeV, C′, C, Y1 and Y2 comprise a nested set with a shared C-terminal. There is less degree of sequence homology in the C protein between SeV and BPIV-3, compared to the high degree of homology (~70%) between SeV and HPIV1 (Nagai, 1999). The C proteins are small basic polypeptides that may be involved in the viral growth cycle, control of viral RNA synthesis (Lamb and Parks, 2007), counteracting host-cell antiviral pathways (Komatsu *et al.*, 2004) and facilitating the release of virus from infected cells (Garcin *et al.*, 1997; Kato *et al.*, 2001). The C protein of BPIV-3 has been shown to suppress double-stranded RNA-stimulated IFN-β production to some degree, possibly through MDA5 or RIG-I mediated signaling because its also inhibits IFN-β production stimulated, not only by TRIF, but also by TBK1 or IKKi downstream of both MDA5 and RIG-I (Komatsu *et al.*, 2007). Therefore, the available evidence suggests the involvement of both V and C proteins in inhibition of type I IFN. However, it is presently unknown whether the BPIV-3 V or C proteins block IFN-β by targeting members of the JAK-STAT pathway.

7.4.4 Matrix protein

The M protein is the most abundant protein in the virion, comprising 341–375 residues with a molecular weight of 38.5–41.5 kDa. The net charge at neutral pH is +14 to 17, making it a basic protein. Although there is no transmembrane domain that has sufficient length to span a lipid bilayer, M protein is somewhat hydrophobic and peripherally associated with membranes (Lamb and Parks, 2007). The M protein is considered to be the central organizer of viral morphogenesis, interacting with the cytoplasmic tails of integral membrane protein, F and HN, the lipid bilayer and the nucleocapsids (Blumberg *et al.*, 1984; Sanderson *et al.*, 1993; Stricker *et al.*, 1994; Cathomen *et al.*, 1998; Schmitt *et al.*, 1999). The so-called 'late domains' have been identified in several paramyxoviruses that interact with cellular protein sorting machinery (Karron and Collins, 2007). The presence or absence of a phosphorylation site in the M protein did not change the phenotype of SeV virions (Sakaguchi *et al.*, 1997).

7.4.5 L protein

The L protein, located at the most promoter distal part in the genome, is the least abundant protein in virus particles. A paramyxovirus particle contains only ~50 copies of L (Lamb *et al.*, 1976). It is an essential subunit of paramyxovirus RNA-dependent RNA polymerase (RdRp). The L gene consists of ~2200 aa with a molecular weight of 220–250 kDa. The L protein possesses all the enzymatic activities needed for polymerization, 5′-end capping, methylation and 3′-end polyadenylation of mRNA (Grdzelishvili *et al.*, 2005; Karron and Collins, 2007; Lamb and Parks, 2007). The L protein associates with P protein to form the active viral polymerase and the polymerase complex can recognize the helical N-RNA template (Hamaguchi *et al.*, 1983; Poch *et al.*, 1990). The L protein of BPIV-3 possesses a conserved domain structure, template recognition, ATP-binding and polymerization sites similar to those of other *Respiroviruses*.

7.5 Viral Glycoproteins

7.5.1 Fusion protein

The F glycoprotein is a type I integral membrane protein mediating viral penetration

by fusion between the virion envelope and the host-cell plasma membrane at neutral pH. The F protein is composed of 540–580 aa residues. The BPIV-3 F protein is 540 residues long. However, the 3′ untranslated regions of BPIV-3 strains SF/Ka differ from strain 910N by being 14-nt shorter (Qiao *et al.*, 2009). The F protein is synthesized as an inactive precursor, F_0. To be biologically functional, F_0 must be proteolytically cleaved to produce the active fusion protein, which consists of disulfide-linked F_1 and F_2 polypeptides (Scheid and Choppin, 1974). This cleavage event is required for progeny virions to become infectious (Nagai *et al.*, 1976; Garten *et al.*, 1980). The cleavage of F_0 is a candidate for a key determinant for infectivity and pathogenicity for certain viruses. The *Paramyxoviridae* has a cleavage signal sequence located at the N-terminal of F with multibasic or single basic residue at the cleavage site. The cleavage of F containing multibasic residues at the cleavage site occurs intracellularly by furin, a subtilisin-like endoprotease (Klenk and Garten, 1994), while F with a single basic residue at the cleavage site must be expressed at the cell surface and incorporated into released virions and then can be cleavage activated by the addition of exogenous protease (Scheid and Choppin, 1974). For NDV, the nature of the cleavage site correlates with the virulence of the virus strains. The ones with multibasic residues at the cleavage site show virulence and readily disseminate through the host, whereas strains with a single basic residue at the cleavage site are avirulent and tend to be restricted to the respiratory and alimentary tracts where the necessary secreted protease is present (Nagai and Klenk, 1977). The C-terminal of F has a hydrophobic transmembrane domain that anchors the protein into the membrane, leaving a short cytoplasmic tail (~20–40 residues). A 4–3 (heptad) pattern of hydrophobic repeats, designated heptad repeats HRA and HRB, can be found between the fusion peptide and the transmembrane domain, approximately 250-aa apart from each other (Lamb and Parks, 2007). Biophysical data and crystallographic studies have shown that HRA and HRB can form a helical hairpin or six-helix bundle (6HB) structure (core trimer) in F protein homotrimers and this 6HB formation is tightly linked to the merger of lipid bilayers and is believed to couple the free energy released on protein refolding to membrane fusion (Melikyan *et al.*, 2000).

7.5.2 Attachment protein

Attachment protein is another integral membrane glycoprotein in *Paramyxoviridae* involved in the cell attachment process, which is also an integral membrane protein (Lamb and Parks, 2007). The attachment protein of *Respirovirus*, *Avulavirus* and *Rubulavirus* binds to cellular sialic acid-containing receptors and are capable of agglutinating erythrocytes. These attachment proteins also have neuraminidase activity that cleaves sialic acid from the progeny virus particles to prevent viral self-aggregation (Scheid and Choppin, 1974) and enable release of virions from the cell membrane. Thus the protein has been designated HN. HN is a type II membrane protein composed of 565–582 residues that span the membrane once. It contains an N-terminal cytoplasmic tail, a single N-terminal transmembrane domain and a membrane-proximal stalk domain that supports a C-terminal globular head domain. The globular head domain contains both receptor binding and enzymatic activity (Hiebert *et al.*, 1985). The attachment protein of BPIV-3 is 1888-nt long with a single open reading frame that could encode a 572-aa residue protein. HN presents on the surface of virions and infects cells as a tetramer consisting of pairs of homodimers (Thompson *et al.*, 1988). BPIV-3 strains SK217 and SF/Ka were shown to differ in virulence (Shibuta *et al.*, 1981; Breker-Klassen *et al.*, 1996). This virulence difference had been associated with a change in amino acid 193 in HN protein, which had a dramatic effect on syncytium-inducing activity, neuraminidase activity and haemagglutinating activity (Breker-Klassen *et al.*, 1996).

For most paramyxoviruses, the receptor binding protein (HN, N or G) is required

to mediate the fusion reaction (Morrison, 2003; Lamb *et al.*, 2006). The precise role of HN/N/G in stimulating the F conformational change remains to be understood. However, HN may execute its function by stabilizing the F pre-fusion stalk in a receptor-dependent manner (Lamb and Parks, 2007). Several models were studied to rationalize the involvement of HN in fusion promotion (Lamb, 1993; Lamb and Parks, 2007). A model in NDV proposed a second sialic acid binding site in addition to the active site (Crennell *et al.*, 2000). The location of this new site at the HN dimer interface, together with observation that changes in the HN structure that appear to be generated by catalysis alter the association of the HN dimer or tetramer and may also propagate a change in the stalk region that triggers the fusion protein, suggests a model for how sialic acid binding by HN might trigger conformational changes in F (Zaitsev *et al.*, 2004).

For all paramyxoviruses, co-expression of F and HN (H or G) is required for fusion or the co-expression of HN makes fusion more efficient (Karron and Collins, 2007). An interaction of F with homotypic and not heterotypic HN has been shown to promote fusion (Horvath *et al.*, 1992; Sergel *et al.*, 1993). The F and HN glycoprotein genes of BPIV-3 were shown to be the major determinants of host-range restriction in rhesus monkeys by generating HPIV3 chimeras with BPIV-3 envelope glycoproteins (Schmidt *et al.*, 2000), but a subsequent study confirmed that the host-range restriction of replication of BPIV-3 is polygenic (Skiadopoulos *et al.*, 2003).

7.6 Antigenic Analysis

The immunity to PIV appears to be short lived (Glezen *et al.*, 1984) and reinfections with the same types may occur within short time intervals (Chanock *et al.*, 1961; Muchmore *et al.*, 1981). This is stated to be the result of poor immunological response in the respiratory tract rather than of antigenic instability (Yanagihara and McIntosh, 1980). The efficacy of post-infection immunity and difference in the virulence of isolates/strains may also depend on antigenic differences (Shibuta *et al.*, 1982, 1985). Plaque variants of BPIV-3 strain YN show differences in virulence in mice (Shibuta *et al.*, 1985). The SC-YN variant and 910N strain induce a non-lethal hydroencephalus when inoculated intracerebrally in newborn mice, but the M-YN variant causes a lethal brain disease (Shibuta *et al.*, 1982). There are very few studies that examined the antigenic characteristics of BPIV-3 employing monoclonal antibodies (MAbs). Human and bovine PIV3 share neutralizing epitopes but show distinct antigenic properties (Muchmore *et al.*, 1981; Glezen *et al.*, 1984; Coelingh *et al.*, 1986). In an analysis with 52 MAbs against N, M, F, HN proteins of PIV3, it was established that the bovine and human PIV3 strains were antigenically quite different (Rydbeck *et al.*, 1987). Six epitope groups in the HN were described (Shibuta *et al.*, 1986; Rydbeck *et al.*, 1987). Epitope III specific HN-MAbs showed no heterotypic reaction to human PIV3 (Klippmark *et al.*, 1990). Fifty-three MAbs to the human C243 strain were directed against six, four, nine and seven epitopes of the HN, F, N and M proteins, respectively. Seven MAbs to the bovine strain 910N were directed against three epitopes of the HN and three epitopes of the F protein. The M protein of BPIV-3 is antigenically conserved and the F protein is equally homogeneous. There appears to be more variation in the N protein and limited variations of HN antigenic epitopes amongst BPIV-3 (Klippmark *et al.*, 1990). Overall, the available evidence suggests that there is only limited antigenic variation among BPIV-3 strains recovered at different geographic regions at various times.

7.7 Transcription/Replication

The PIV ribonucleoprotein (RNP) or transcriptase complex, consisting of the negative sense genomic RNA encapsidated with N protein, and the associated RdRp heterocomplex, the L and P proteins, is released into the cytoplasm following fusion of

the cell membrane with the viral envelope (Chattopadhyay *et al.*, 2011). The N-RNA is used by the RdRp as a template for transcription and replication in the presence of P protein co-factor activity. It is generally believed that all negative sense, non-segmented RNA viruses (NSV) including PIV follow a common transcription and replication strategy (Banerjee *et al.*, 1977; Karron and Collins, 2007; Lamb and Parks, 2007; Chattopadhyay *et al.*, 2011). The standard scheme for transcription and replication of NSV at present is as follows: the RdRp heterocomplex enters the viral genome at the 3′ end and initiates transcription to synthesize the leader RNA and viral mRNAs by a start–stop mechanism (Emerson, 1982). The RdRp also is responsible for capping, cap methylation and addition of polyA tail at the 3′ end of the nascent mRNA (Ogino and Banerjee, 2007). Re-initiation of transcription at the gene-start site of NSV is imperfect, which leads to a gradient of mRNA abundance that decreases down the viral genome, depending on the distance from the 3′ end of the viral genome. This property is known as 'polarity of transcription' (Fig. 7.3). Several host proteins such as cellular actin, tubulin (Moyer *et al.*, 1986; Ogino *et al.*, 2003), β catenin (Bose and Banerjee, 2004), and cellular glycolytic enzymes, phosphoglycerate kinase and enolase (Ogino *et al.*, 2001), and specific phosphorylated forms of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Choudhary *et al.*, 2000) have been shown to be involved in the transcription and replication of PIV. The switch from transcription to replication is unclear. However, when a sufficient amount of N protein accumulates in the cell, it complexes with the P protein to form a soluble N–P complex, which is used for the replication of the progeny genome RNA. Replication commences when the RdRp ignores the transcription stop signals at each gene junction and a full-length positive sense antigenome is synthesized. The anti-genome is also fully encapsidated by the nucleocapsid proteins and the anti-genome then serves as a template for the synthesis of the negative sense genome (Fig. 7.3). It is surmised that the antigenomic promoter is stronger than the genomic

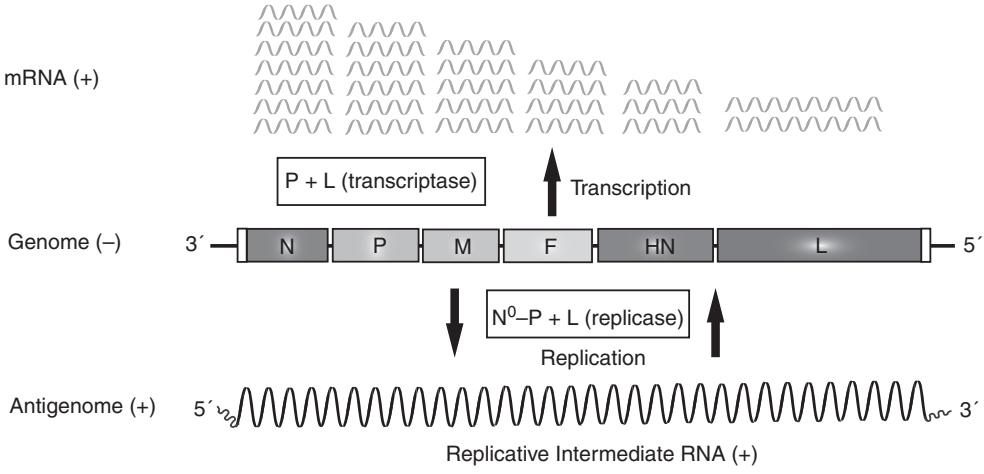


Fig. 7.3. Schematic of the general replication strategy of PIVs. The virion-encoded RNA-dependent RNA polymerase (RdRp), consisting of the L protein with its co-factor P protein, commences the primary transcription of the negative-sense encapsidated RNA genome (N-RNA) of PIVs in the cytoplasm. The leader RNA and the mRNAs are produced by a start–stop mechanism. The 'polarity of transcription' is shown as decreasing quantities of mRNA distal to the 3′ end of the genome. When sufficient N protein is accumulated in the cytoplasm, it complexes with the P protein to form a soluble (N^0–P) complex, and the polymerase switches from transcriptase to replicase mode to make the full-length anti-genomic strand, which in turn is copied to produce the (–) sense RNA genome.

promoter, resulting in more copies of the genome sense RNA, which is encapsidated, packaged and released as virions. There are excellent recent reviews on the complex mechanisms of transcription and replication in NSV (Karron and Collins, 2007; Lamb and Parks, 2007; Banerjee, 2008; Whelan, 2008; Chattopadhyay *et al.*, 2011).

7.8 Pathogenesis

BPIV-3 is primarily a respiratory tract pathogen (Bryson *et al.*, 1983a). The primary portal of entry of BPIV-3 is through the respiratory tract via virus-laden droplets. The complexity of determining each of the subsequent steps post-adsorption of the virus to the respiratory mucosa is compounded by the fact that most BPIV-3 infections occur in combination with other agents. However, most experimental studies that used aerosol, intranasal or combined intranasal and intratracheal routes of inoculation in young calves suggest that once the virus is established in the respiratory tract, a transient viraemia may ensue (Dawson *et al.*, 1965; Woods *et al.*, 1965), followed by a transient tissue residence of the virus in extrapulmonary tissues (Van der Maaten, 1969). Enteritis (Hamdy, 1966) or splenitis (Woods *et al.*, 1965) are occasionally associated with BPIV-3 infection. The reader is referred to a recent review (Ellis, 2010) for an excellent description of BPIV-3 infection at the cellular level. However, at present, the nature of cell injury inflicted by BPIV-3 *in vivo* is unclear. Bryson *et al.* (1983b) made the first detailed ultrastructural description of BPIV-3 infection in cells of the respiratory tract. Virus replication occurs in the cytoplasm as in other NSV. Viral nucleocapsid aggregates were observed in the ciliated cells of the upper respiratory tract, ciliated and nonciliated bronchiolar cells, in type I and type II pneumocytes and in a fraction of alveolar macrophages. Nucelocapsids and budding viral particles were observed most commonly in the first 7 days post-infection (Bryson *et al.*, 1983b). Acute BPIV-3 infection leads to destruction and loss of cilia and of ciliated cells and hyperplasia of type II pneumocytes (Bryson *et al.*, 1983b), possibly resulting in increased surfactant secretion and limited air exchange. Virus-infected calf alveolar macrophages were shown to have decreased cytotoxic capabilities for virus-infected cells (Probert and Lwin, 1976). Several studies demonstrated that BPIV-3 infection results in depressed phagocytosis and bacterial cell killing ability of calf alveolar macrophages (Liggitt *et al.*, 1985; Slauson *et al.*, 1987; Brown and Ananaba, 1988). Defects in neutrophil function due to BPIV-3 infection have also been suggested (Briggs *et al.*, 1988). The destruction of ciliated and nonciliated cells of the respiratory tract along with defects in the mucociliary apparatus and functional defects in innate immune cells suggests that BPIV-3 infection lowers the innate immune defence of the respiratory tract, paving the way for the secondary infections.

As most infections with BPIV-3 are complicated by the presence of other pathogens, the actual damage induced by the virus in naïve cattle under natural settings is unclear. However, several experimental studies conducted from the late 1960s to the 1980s in seronegative and seropositive calves highlight the ability of the virus to induce pathology in the respiratory tract. The extent of tissue damage and virus shedding depend on the virulence of the infecting virus strain, the magnitude and duration of challenge, the immune status of the animal and environmental stress factors. Most severe respiratory tissue damage was evident in young seronegative calves infected with certain virulent strains of BPIV-3 (Dawson *et al.*, 1965; Omar *et al.*, 1966; Tsai and Thomson, 1975; Bryson *et al.*, 1978). Calves with circulating maternal antibodies to BPIV also shed virus upon experimental infection (Marshall and Frank, 1975; Bergman, 1978). With a single exposure to the virus, shedding has been demonstrated for up to 8–10 days post-infection (Frank and Marshall, 1971). But with repeated virus exposures as expected in field conditions, virus shedding could not be detected 12 days post-infection (Bryson *et al.*, 1979). Reinfection of seropositive calves after several

weeks of primary exposure was shown to result in shorter duration of virus shedding (Frank and Marshall, 1971). Interestingly, in naturally occurring BPIV-3 infection, virus has been found in the respiratory tract of individual calves for several months (Stott *et al.*, 1975; Burgess, 1977; Allan *et al.*, 1978). It appears that under natural settings, susceptible cattle would be exposed to continuous challenge with BPIV-3. It is unclear whether there is persistent infection of infected cattle, because no experimental studies exist to prove this.

7.9 Clinical Signs

Infection with BPIV-3 is global in beef and dairy cattle 2–8 months old, but clinical disease due to primary BPIV-3 infection is rare. Most isolations of BPIV-3 are from undifferentiated bovine respiratory disease complex, where multiple infectious agents complicate the clinical disease. Even in experimental infections with BPIV-3, the severity of clinical disease varies considerably. Subclinical disease or mild clinical disease with coughing, transient fever, mild depression, increased respiratory rate and oculonasal discharges are the most common findings (Frank and Marshall, 1971; Allan *et al.*, 1978). Occasionally, moderate to severe respiratory illness characterized by marked tachypnoea, hyperpnoea and pneumonia has been described (Omar *et al.*, 1966; Bryson *et al.*, 1989). Experimentally, BPIV-3 has also been shown to produce systemic disease in calves (Reisinger *et al.*, 1959; Dawson *et al.*, 1965; Woods *et al.*, 1965) and extensive pneumonia in both lambs (Hore and Stevenson, 1969; Biberstein *et al.*, 1971) and calves (Dawson *et al.*, 1965; Omar *et al.*, 1966). Outbreaks of respiratory disease in adult cattle with nasal erosions, fibrinous casts, epistaxis and submandibular oedema have been reported (Coswell and Williams, 2007). BPIV-3 has been shown to be associated with a variety of disease syndromes, including UF/BRDC and enzootic pneumonia of calves. The average cost of one treatment for UF/BRDC in the USA was estimated at US$15.57 (Faber *et al.*, 1999). This cost rises to US$92.26 (McNeil *et al.*, 1996) when indirect costs are also considered, such as the reduction of average daily gain and a lower carcass value because of a less desirable quality grade in feedlot cattle. The incidence rate of clinical UF/BRDC was reported as 14.4% (USDA-APHIS, 2001) from a study of feedlots from 12 states in 1999. Lung lesions at slaughter in feedlot cattle are extremely common, with an observed prevalence ranging between 29.7 and 77% (Wittum *et al.*, 1996; Bryant *et al.*, 1999; Thompson *et al.*, 2006).

7.10 Pathology

The documentation of experimental and natural BPIV-3 infection in cattle and other species is sparse. From those studies where gross pathology was described, it could be gleaned that in natural cases of primary BPIV-3, pneumonia is mild and consists of a cranioventral or generalized, lobular pattern of grey-red discoloration, firm or rubbery texture, and mild swelling or atelectasis of lung tissue (Coswell and Williams, 2007). Emphysema of the caudal lung lobes in calves that died of respiratory distress has been described (Coswell and Williams, 2007). In experimental infections, the gross pathology varied from mild rhinitis and tracheobronchitis to mild to extensive pneumonia (Dawson *et al.*, 1965; Omar *et al.*, 1966; Bryson *et al.*, 1979).

Bronchioloitis and mild bronchitis are the major histologic lesions in natural cases of uncomplicated primary BPIV-3 pneumonia (Coswell and Williams, 2007). In the upper respiratory tract, epithelial hyperplasia, metaplasia or necrosis with cellular infiltrate of mono- and polynuclear cells are observed (Bryson *et al.*, 1983a). In the lower respiratory tract, the virus induces pathology in small bronchi, bronchioles and alveoli. Epithelial cells lining small bronchioles appear rounded, occasionally vacuolated, and slough into the lumen occluding the airways (Bryson *et al.*, 1983a; Coswell and Williams, 2007). The epithelial layer may

be discontinuous, attenuated or hyperplastic (Coswell and Williams, 2007). In the acute stages of BPIV-3 infection, eosinophilic intracytoplasmic and sometimes intranuclear inclusion bodies in many bronchial, bronchiolar and alveolar epithelial cells and alveolar macrophages (Bryson *et al.*, 1983a; Coswell and Williams, 2007) are observed. In experimentally infected calves, inclusions are most common from 2 to 4 days and less common from 4 to 5 days post infection. Epithelial syncytial formation on bronchiolar and alveolar walls has been reported as a prominent feature of infections with some strains of BPIV-3 (Dawson *et al.*, 1965; Omar *et al.*, 1966; Bryson *et al.*, 1979), but is less common than in bovine respiratory syncytial virus infection. Mildly oedematous bronchiolar walls are infiltrated by low numbers of neutrophils and lymphocytes. Alveoli are atelectatic, oedematous and with increased numbers of macrophages and neutrophils (Bryson *et al.*, 1983a; Coswell and Williams, 2007). Experimental infections with high doses of virus result in hyperplasia of type II pneumocytes and thickening of alveolar septa. Organization of bronchiolar exudate may lead to bronchiolitis obliterans with fibrous masses (Bryson *et al.*, 1983a).

7.11 Immunity

7.11.1 Innate immunity

The innate immune recognition of PIV is primarily made by pattern-recognition receptors (PRR) viz. retinoic acid-inducible gene I (RIG-I)-like receptors (RLR including RIG-I and MDA5) and toll-like receptors (TLR) (Takeuchi and Akira, 2009). Type I interferon, cytokines and interferon-inducible genes like protein kinase R (PKR) and 2′, 5′-oligoadenylate synthase are produced by RLR and TLR upon virus infection (Takeuchi and Akira, 2009). The activation of RLR and TLR by SeV, a prototype *Respirovirus* and NDV, a prototype *Avulavirus*, induces type I IFN and cytokine depending on the cell type (Melchjorsen *et al.*, 2005; Biswas *et al.*, 2012). This property is also shared by other negative sense RNA viruses (Takeuchi and Akira, 2009). Recently, it was reported that RIG-I could act as a PRR for HPIV3 (Sabbah and Bose, 2009). As RIG-I recognizes the uncapped, 5′-triphosphate (5′-ppp)-ended RNA produced in NSV-infected cells, BPIV-3 is more likely to induce type I IFN and IFN-inducible genes similar to other NSV. However, from the available evidence based on the ability of BPIV-3 V protein to block IFN-β by targeting MDA5 and C protein by targeting MDA5, TRIF, TBK1, IKKi (Komatsu *et al.*, 2007), it can be stated that BPIV-3 stimulates RLR through 5′-ppp, but the molecular mechanisms of IFN induction through MDA5 is unclear for BPIV-3 and other PIV.

While innate immune recognition of PIV infection by non-immune cells depends mostly on RLR, in some specialized immune cells virus recognition is mediated by TLRs (Melchjorsen *et al.*, 2005). For a review of TLRs, a class of membrane-bound PRR on the cell surface or endosome, and their role in recognizing RNA viruses, the reader is referred to recent publications (Brennan and Bowie, 2010; Aoshi *et al.*, 2011). Recently, the L protein of PIV5 has been shown to interact with Akt1, which can activate NF-κB, upon phosphorylation leading to the production of pro-inflammatory cytokines (Luthra *et al.*, 2008). The role of C-type lectin receptors (Geijtenbeek and Gringhuis, 2009), Nod-like receptors (NLRs) (Franchi *et al.*, 2009) and AIM2-like receptors (ALRs) (Unterholzner *et al.*, 2010) in the recognition of PIV awaits future research.

7.11.2 Adaptive immunity

Most adult bovines have antibodies to BPIV-3 and therefore calves will obtain maternal antibodies from their dams. These maternal antibodies generally persist for up to 10 weeks and in those that received a high initial level of colostral antibodies up to 19–23 weeks of age (Dawson, 1966). The disease is less severe in colostrum-fed calves than colostrum-deprived calves,

although both groups became infected, shed virus and had clinical disease after aerosol exposure (Omar *et al.*, 1966; Marshall and Frank, 1975; Bryson *et al.*, 1979). Colostral antibodies also drained to the nasal compartment and protect from clinical disease (McKercher *et al.*, 1972). The serum and nasal antibody responses of colostrum-fed calves upon aerosol challenge with BPIV-3 were depressed compared with seronegative calves, suggesting maternal antibodies interfered with adaptive immunity (Marshall and Frank, 1975). Both local and systemic virus-neutralizing antibodies of the IgG and IgM type (Morein, 1970; McKercher *et al.*, 1972; Marshall and Frank, 1975) and mucosal (nasal) IgA type (Morein, 1970) are produced after infection with BPIV-3. The serum antibodies have been shown to persist for 3–5 months (Dawson *et al.*, 1965; Marshall and Frank, 1971), but nasal antibodies declined rapidly within 6–8 weeks (Frank and Marshall, 1971). Similarly, after reinfection, the serum antibodies persisted for 5 months, but the nasal antibodies fell rapidly (Frank and Marshall, 1971). Calves with low levels of nasal antibody but high level of serum antibodies after reinfection were not protected after challenge, while calves with high levels of nasal antibodies but low levels of serum antibody were protected (Frank and Marshall, 1971). This suggests that nasal antibodies prevent infection, while serum antibodies protect against severe clinical disease once infected. Reduced viral shedding was also noticed in calves with high levels of nasal secretory antibodies (Gates *et al.*, 1970).

The role of cell-mediated immune response against BPIV-3 in protecting against clinical disease is unclear due to lack of detailed studies. Delayed type hypersensitivity reactions in infected (seropositive) calves, but not in maternal antibody-positive calves, to BPIV-3 antigens have been reported (Morein and Moreno-Lopez, 1973). Antigen-specific lymphocyte proliferation and leukocyte migration inhibition against BPIV-3 antigens in infected cattle have been demonstrated (Johnson and Morein, 1977; Moreno-Lopez, 1977). Macrophages recovered from calves by bronchoalveolar lavage appear to retain all their biological functions and are capable of mediating cytotoxicity against BPIV-3-infected cells (Stott *et al.*, 1975; Bradford *et al.*, 1992). BPIV-3 infected cell lysis independent of MHC restriction through NK-like cells (Campos *et al.*, 1982), and also by MH-restricted cytotoxic T cells 6–9 days post-infection (Bamford *et al.*, 1995) have been reported. Antibody-independent killing of BPIV-3 virus-infected cells was observed with neutrophils, alveolar macrophages and lymphocytes (Bradford *et al.*, 1992). Levels of specific lysis up to 30% for neutrophils and 68% for alveolar macrophages were observed, although there was considerable variation in activity from animal to animal. Lymphocyte preparations showed levels of cytotoxicity up to 20% in some cases, while monocytes had low killing ability (Bradford *et al.*, 1992). Addition of BPIV-3 virus-specific antibodies enhanced killing by neutrophils, monocytes and lymphocytes (Bradford *et al.*, 1992), but inhibited killing by alveolar macrophages (Campos *et al.*, 1982; Bradford *et al.*, 1992; Adair *et al.*, 1999). Following intranasal and intratracheal inoculation of calves with BPIV-3 virus, the level of antibody-independent cytotoxicity by macrophages lavaged from the lungs of the calves increased substantially, and by day 5 post-inoculation, but declined rapidly after 5 days (Adair *et al.*, 1999). Complement, particularly guinea pig complement, was cytotoxic for virus-infected but not for uninfected cells, and also considerably enhanced the cytotoxic effect of neutrophils and lymphocytes (Bradford *et al.*, 1992).

7.12 Diagnosis

It is difficult to diagnose BPIV-3 infection in an outbreak of bovine respiratory disease based on clinical signs. The confirmatory diagnosis of BPIV-3 is frequently made by isolation and identification of the virus and demonstration of increasing antibody titres using paired sera. The most commonly employed serological tests include

haemagglutination-inhibition and virus neutralization. For ante-mortem diagnosis by virus isolation, nasal discharges collected using Dacron swabs in transport medium are preferred to conventional cotton-tipped swabs because the latter is a poor source of material for virus isolation (Thomas and Stott, 1975). Nasal mucus collected using vacuum pumps offers the convenience of providing not only excellent material for virus isolation but also for rapid immunohistochemical diagnosis because of the presence of epithelial cells (McFerran and McNulty, 1981). Samples must be collected early in the course of the disease from a number of animals in the herd showing a range of clinical signs. BPIV-3 is extremely labile in storage and transport and therefore the samples should be submitted to the diagnostic laboratory as soon as possible. From necropsied animals, fresh or frozen lung tissues harvested from the cranioventral areas of lung with lesions are ideal specimens for virus isolation and antigen detection tests. Fluorescent antibody tests on frozen lung tissue and immunohistochemistry on formalin-fixed lung tissue are commonly employed diagnostic tests. BPIV-3 infection should be considered in calves with necrotizing bronchiolitis. However, post-mortem antigen detection and virus isolation tests are effective in acute cases, but invariably ineffective in most common necropsy presentations of complicated BRDC. Confirmatory diagnosis in these cases may prove difficult because the virus or viral antigens may be cleared long before the fatal bacterial pneumonia (Coswell and Williams, 2007). Reverse-transcriptase polymerase chain reaction (RT-PCR) and quantitative real-time RT-PCR techniques may be employed on nasal swabs, nasal mucus and lung tissues. Because of the multifactorial aetiology of UF/BRDC, the high incidence of subclinical BPIV-3 infections and the use of modified live virus BPIV-3 vaccines, detecting the virus, viral antigens or viral nucleic acid does not prove disease causation. Therefore, the test results should be interpreted after taking into consideration the overall clinical condition of the herd and the individual animal (MacLachlan and Dubovi, 2011).

7.13 Prophylaxis and Control

Several studies demonstrated that environmental factors, including poor ventilation in calf houses, dusty environments and stress in feedlot calves, increase the severity of BPIV-3-associated respiratory disease in cattle (Woods *et al.*, 1968; Frank and Marshall, 1971). Therefore, it is imperative that adequate hygiene precautions and enhanced general husbandry practices should be major goals for preventing and reducing transmission of BPIV-3 in beef and dairy cattle.

Inactivated and live-attenuated virus vaccines for intranasal and parenteral administration are currently available that induce protective antibodies. Currently available vaccines in the market are combination vaccines that are formulated with a variety of antigens including bovine herpes virus 1, bovine respiratory syncytial virus, bovine viral diarrhoea virus and *Mannheimia hemolytica*. The intranasal BPIV-3 vaccines are temperature-sensitive and restricted for replication in the cooler temperatures of the bovine nasal passages (Vangeel *et al.*, 2009). The efficacy of the current vaccines, irrespective of the formulation, is fair in dairy cattle, but with the different management issues confronting the feedlot industry, the control of UF/BRDC is complicated using the vaccination approach. There are conflicting reports on the efficacy of the intranasal vaccine formulations in comparison with parenteral live attenuated vaccines (Ellis, 2010). However, both types of vaccine are capable of inducing local and systemic protective antibody responses, but the duration and magnitude of response may depend on the status of local and systemic antibody levels at the time of vaccination, as discussed earlier. Maternal antibodies inhibit the development of active immunity against BPIV-3 (Marshall and Frank, 1975; Adair *et al.*, 2000; Fulton *et al.*, 2004). Maternal antibody virus neutralization

titres between 1:8 and 1:16 before vaccination would result in 90% of the calves responding positively to parenteral vaccination by day 28 and a titre of 1:4 before vaccination would allow a better response by 14 days after vaccination (Fulton *et al.*, 2004). Using a commercial ELISA and relative optical density values (ROD) against anti-BPIV-3 IgG, it was determined that for the BPIV-3 antibody responses on days 14, 28 and 42, the 90% positive ROD thresholds (VN titre equivalent) were 1.5% (1:4.6), 11.6% (1:8.5) and 14.3% (1:9.3), respectively (O'Neill *et al.*, 2007).

Although there is some evidence that intranasal vaccine may increase macrophage chemotaxis in the lungs (Adair *et al.*, 2000), there is not enough evidence to show superiority of intranasal over conventional parenteral vaccines. Using mouse models, nanoparticle-encapsulated intranasal BPIV-3 vaccines were shown to induce higher levels of antibodies (Shephard *et al.*, 2003). However, the efficacy of this approach awaits field trials in cattle. The difficulty in assessing the efficacy of various vaccine formulations because of inconsistencies in the challenge models, metrics for potency and the inevitable virus shedding even in vaccinated animals upon challenge were reviewed recently (Ellis, 2010). A recent study suggested that in a population of commercial, home-raised dairy calves, with an overall low incidence of failure of passive transfer, intramuscular vaccination with a multivalent, modified live viral vaccine at 2 or 5 weeks of age or both was not associated with a decreased risk of BRDC or mortality, or with body weight gain until 3 months of age (Windeyer *et al.*, 2012). These results were attributed to interference by maternal antibodies, unresponsiveness of the neonatal immune system, timing of immunity relative to pathogen exposure, disease caused by pathogens other than the viruses in the vaccine, or herd immunity. However, in populations with higher incidence of failure of passive transfer or risk of BRDC, calves with low levels of specific antibodies may respond differently to vaccination (Windeyer *et al.*, 2012). The use of TLR agonists as adjuvants to respiratory vaccines is being explored. Synthetic oligodeoxynucleotides containing CpG motifs that signal through TLR9 and activate innate immunity were tested on PIV-3 nasal shedding in lambs and it was found that pre-treatment of lambs 2 days but not 5 days before viral challenge reduced nasal shedding (Nichani *et al.*, 2010). Although these approaches are promising, effective vaccines against BPIV-3 that would be able to invade high levels of maternal antibodies, provide longer duration of immunity and reduce nasal virus shedding are yet to be developed. Until then, sound management practices depending on the type of rearing and early achievement of protective mucosal immune response should be the main goals of prevention and control of this insidious respiratory pathogen.

References

Adair, B.M., Bradford, H.E., McNulty, M.S. and Foster, J.C. (1999) Cytotoxic interactions between bovine parainfluenza type 3 virus and bovine alveolar macrophages. *Veterinary Immunology Immunopathology* 67, 285–294.

Adair, B.M., Bradford, H.E., Bryson, D.G., Foster, J.C. and McNulty, M.S. (2000) Effect of parainfluenza-3 virus challenge on cell-mediated immune function in parainfluenza-3 vaccinated and non-vaccinated calves. *Research in Veterinary Science* 68, 197–199.

Allan, E.M., Pirie, H.M., Selman, I.E. and Snodgrass, D.R. (1978) Some characteristics of a natural infection by parainfluenza-3 virus in a group of calves. *Research in Veterinary Science* 24, 339–346.

Andrejeva, J., Poole, E., Young, D.F., Goodbourn, S. and Randall, R.E. (2002) The p127 subunit (DDB1) of the UV-DNA damage repair binding protein is essential for the targeted degradation of STAT1 by the V protein of the paramyxovirus simian virus 5. *Journal of Virology* 76, 11,379–11,386.

Andrejeva, J., Childs, K.S., Young, D.F., Carlos, T.S., Stock, N., Goodbourn, S. and Randall, R.E. (2004) The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter. *Proceedings of the National Academy of Sciences USA* 101, 17,264–17,269.

Andrewes, C.H., Bang, F.B., Chanock, R.M. and Zhdanov, V.M. (1959) Para-influenza viruses 1, 2, and 3: suggested names for recently described myxoviruses. *Virology* 8, 129–130.

Aoshi, T., Koyama, S., Kobiyama, K., Akira, S. and Ishii, K.J. (2011) Innate and adaptive immune responses to viral infection and vaccination. *Current Opinions in Virology* 1, 226–232.

Bamford, A.I., Adair, B.M. and Foster, J.C. (1995) Primary cytotoxic response of bovine peripheral blood leukocytes to parainfluenza type 3 virus infection. *Veterinary Immunology Immunopathology* 45, 85–95.

Banerjee, A.D., Abraham, G. and Colonno, R.J. (1977) Vesicular stomatitis virus: mode of transcription. *Journal of General Virology* 34, 1–8.

Banerjee, A.K. (2008) Response to 'Non-segmented negative-strand RNA virus RNA synthesis in vivo'. *Virology* 371, 231–233.

Baron, M.D. and Barrett, T. (2000) Rinderpest viruses lacking the C and V proteins show specific defects in growth and transcription of viral RNAs. *Journal of Virology* 74, 2603–2611.

Bergman, R. (1978) Parainfluenza-3 virus: difference in capacity of neuraminidase-weak and strong strains to infect young calves and to elicit cellular immune response. *Research in Veterinary Science* 25, 193–199.

Bhella, D., Ralph, A., Murphy, L.B. and Yeo, R.P. (2002) Significant differences in nucleocapsid morphology within the Paramyxoviridae. *Journal of General Virology* 83, 1831–1839.

Biberstein, E.L., Shreeve, B.J., Angus, K.W. and Thompson, D.A. (1971) Experimental pneumonia of sheep. Clinical, microbiological and pathological responses to infection with Myxovirus parainfluenza 3 and *Pasteurella haemolytica*. *Journal of Comparative Pathology* 81, 339–351.

Biswas, M., Kumar, S.R., Allen, A., Yong, W., Nimmanapalli, R., Samal, S.K. and Elankumaran, S. (2012) Cell-type-specific innate immune response to oncolytic Newcastle disease virus. *Viral Immunology* 25, 268–276.

Blumberg, B.M., Rose, K., Simona, M.G., Roux, L., Giorgi, C. and Kolakofsky, D. (1984) Analysis of the Sendai virus M gene and protein. *Journal of Virology* 52, 656–663.

Borden, E.C., Sen, G.C., Uze, G., Silverman, R.H., Ransohoff, R.M., Foster, G.R. and Stark, G.R. (2007) Interferons at age 50: past, current and future impact on biomedicine. *Nature Reviews Drug Discovery* 6, 975–990.

Bose, S. and Banerjee, A.K. (2004) Beta-catenin associates with human parainfluenza virus type 3 ribonucleoprotein complex and activates transcription of viral genome RNA in vitro. *Gene Expression* 11, 241–249.

Bradford, H.E., Adair, B.M., McNulty, M.S. and Foster, J.C. (1992) Cytotoxicity of bovine leucocytes for parainfluenza type-3 virus-infected cells. *Veterinary Immunology Immunopathology* 31, 115–127.

Breker-Klassen, M.M., Yoo, D. and Babiuk, L.A. (1996) Comparisons of the F and HN gene sequences of different strains of bovine parainfluenza virus type 3: relationship to phenotype and pathogenicity. *Canadian Journal of Veterinary Research* 60, 228–236.

Brennan, K. and Bowie, A.G. (2010) Activation of host pattern recognition receptors by viruses. *Current Opinions in Microbiology* 13, 503–507.

Briggs, R.E., Kehrli, M. and Frank, G.H. (1988) Effects of infection with parainfluenza-3 virus and infectious bovine rhinotracheitis virus on neutrophil functions in calves. *American Journal of Veterinary Research* 49, 682–686.

Brown, T.T. Jr and Ananaba, G. (1988) Effect of respiratory infections caused by bovine herpesvirus-1 or parainfluenza-3 virus on bovine alveolar macrophage functions. *American Journal of Veterinary Research* 49, 1447–1451.

Bryant, L.K., Perino, L.J., Griffin, D.D., Doster, A.R. and Wittum, T.E. (1999) A method for recording pulmonary lesions of beef calves at slaughter, and the association of lesions with average daily gain. *The Bovine Practitioner* 33, 163–173.

Bryson, D.G., McFerran, J.B., Ball, H.J. and Neill, S.D. (1978) Observations on outbreaks of respiratory disease in housed calves – (2) Pathological and microbiological findings. *Veterinary Record* 103, 503–509.

Bryson, D.G., McFerran, J.B., Ball, H.J. and Neill, S.D. (1979) Observations on outbreaks of respiratory disease in calves associated with parainfluenza type 3 virus and respiratory syncytial virus infection. *Veterinary Record* 104, 45–49.

Bryson, D.G., McNulty, M.S., McCracken, R.M. and Cush, P.F. (1983a) Ultrastructural features of experimental parainfluenza type 3 virus pneumonia in calves. *Journal of Comparative Pathology* 93, 397–414.

Bryson, D.G., McNulty, M.S., Logan, E.F. and Cush, P.F. (1983b) Respiratory syncytial virus pneumonia in young calves: clinical and pathologic findings. *American Journal of Veterinary Research* 44, 1648–1655.

Bryson, D.G., McNulty, M.S., Evans, R.T. and Allan, G. (1989) Studies of the effect of recombinant human-alpha 1 interferon on experimental parainfluenza type 3 virus infections of the respiratory tract of calves. *Veterinary Record* 125, 615–618.

Burgess, G.W. (1977) A study of endemic viruses in a group of calves. *New Zealand Veterinary Journal* 25, 178–179.

Calain, P. and Roux, L. (1993) The rule of six, a basic feature for efficient replication of Sendai virus defective interfering RNA. *Journal of Virology* 67, 4822–4830.

Campos, M., Rossi, C.R. and Lawman, M.J. (1982) Natural cell-mediated cytotoxicity of bovine mononuclear cells against virus-infected cells. *Infection and Immunity* 36, 1054–1059.

Cathomen, T., Naim, H.Y. and Cattaneo, R. (1998) Measles viruses with altered envelope protein cytoplasmic tails gain cell fusion competence. *Journal of Virology* 72, 1224–1234.

Chanock, R.M., Kim, H.W., Vargosko, A.J., Deleva, A., Johnson, K.M., Cumming, C. and Parrott, R.H. (1961) Respiratory syncytial virus. I. Virus recovery and other observations during 1960 outbreak of bronchiolitis, pneumonia, and minor respiratory diseases in children. *Journal of American Medical Association* 176, 647–653.

Chattopadhyay, S., Esper, F. and Banerjee, A.K. (2011) Parainfluenzaviruses. In: Samal, S.K. (ed.) *The Biology of Paramyxoviruses*. Caister Academic Press, Norfolk, UK, pp. 175–209.

Choudhary, S., De, B.P. and Banerjee, A.K. (2000) Specific phosphorylated forms of glyceraldehyde 3-phosphate dehydrogenase associate with human parainfluenza virus type 3 and inhibit viral transcription in vitro. *Journal of Virology* 74, 3634–3641.

Coelingh, K.J., Winter, C.C., Murphy, B.R., Rice, J.M., Kimball, P.C., Olmsted, R.A. and Collins, P.L. (1986) Conserved epitopes on the hemagglutinin-neuraminidase proteins of human and bovine parainfluenza type 3 viruses: nucleotide sequence analysis of variants selected with monoclonal antibodies. *Journal of Virology* 60, 90–96.

Coswell, J.L. and Williams, K.J. (2007) Bovine parainfluenza virus 3. In: Grant Maxie, M. (ed.) *Jubb Kennedy and Palmer's Pathology of Domestic Animals*, vol. 2. Saunders Elsevier, Philadelphia, Pennsylvania, pp. 598–599.

Crennell, S., Takimoto, T., Portner, A. and Taylor, G. (2000) Crystal structure of the multifunctional paramyxovirus hemagglutinin-neuraminidase. *Nature Structural Biology* 7, 1068–1074.

Curran, J.A., Quinn, J.P., Hoey, E.M., Martin, S.J. and Rima, B.K. (1985) cDNA cloning of the nucleocapsid and nucleocapsid-associated protein genes of mumps virus. *Journal of General Virology* 66, 977–985.

Curran, J., de Melo, M., Moyer, S. and Kolakofsky, D. (1991) Characterization of the Sendai virus V protein with an anti-peptide antiserum. *Virology* 184, 108–116.

Curran, J., Marq, J.B. and Kolakofsky, D. (1995) An N-terminal domain of the Sendai paramyxovirus P protein acts as a chaperone for the NP protein during the nascent chain assembly step of genome replication. *Journal of Virology* 69, 849–855.

Dawson, P.S. (1966) Persistence of maternal antibodies to parainfluenza 3 virus. *Journal of Comparative Pathology* 76, 373–378.

Dawson, P.S., Darbyshire, J.H. and Lamont, P.H. (1965) The inoculation of calves with parainfluenza 3 virus. *Research in Veterinary Sci*ence 6, 108–113.

Delenda, C., Hausmann, S., Garcin, D. and Kolakofsky, D. (1997) Normal cellular replication of Sendai virus without the trans-frame, nonstructural V protein. *Virology* 228, 55–62.

Durbin, A.P., McAuliffe, J.M., Collins, P.L. and Murphy, B.R. (1999) Mutations in the C, D, and V open reading frames of human parainfluenza virus type 3 attenuate replication in rodents and primates. *Virology* 261, 319–330.

Edwards, T.A. (2010) Control methods for bovine respiratory disease for feedlot cattle. *Veterinary Clinics of North America Food Animal Practice* 26, 273–284.

Egelman, E.H., Wu, S.S., Amrein, M., Portner, A. and Murti, G. (1989) The Sendai virus nucleocapsid exists in at least four different helical states. *Journal of Virology* 63, 2233–2243.

Eisa, M., Karrar, A.E. and Abdel Rahim, A.H. (1979) The occurrence of antibodies to parainfluenza3 virus in sera of some domestic animals of the Sudan. *British Veterinary Journal* 135, 192–197.

Ellis, J.A. (2010) Bovine parainfluenza-3 virus. *Veterinary Clinics of North America Food Animal Practice* 26, 575–593.

Emerson, S.U. (1982) Reconstitution studies detect a single polymerase entry site on the vesicular stomatitis virus genome. *Cell* 31, 635–642.

Faber, R., Hartwig, N., Busby, W.D. and BreDahl, R. (1999) The costs and predictive factors of bovine respiratory disease in standardized steer tests. *1999 Beef Research Report*. A S Leaflet R1648. Iowa State University, Ames, IA.

Fischer-Tenhagen, C., Hamblin, C., Quandt, S. and Frolich, K. (2000) Serosurvey for selected infectious disease agents in free-ranging black and white rhinoceros in Africa. *Journal of Wildlife Diseases* 36, 316–323.

Franchi, L., Warner, N., Viani, K. and Nunez, G. (2009) Function of Nod-like receptors in microbial recognition and host defense. *Immunological Reviews* 227, 106–128.

Frank, G.H. and Marshall, R.G. (1971) Relationship of serum and nasal secretion-neutralizing antibodies in protection of calves against parainfluenza-3 virus. *American Journal of Veterinary Research* 32, 1707–1713.

Fukuhara, N., Huang, C., Kiyotani, K., Yoshida, T. and Sakaguchi, T. (2002) Mutational analysis of the Sendai virus V protein: importance of the conserved residues for Zn binding, virus pathogenesis, and efficient RNA editing. *Virology* 299, 172–181.

Fulton, R.W. (2009) Bovine respiratory disease research (1983–2009) *Animal Health Research Reviews* 10, 131–139.

Fulton, R.W., Briggs, R.E., Payton, M.E., Confer, A.W., Saliki, J.T., Ridpath, J.F., Burge, L.J. and Duff, G.C. (2004) Maternally derived humoral immunity to bovine viral diarrhea virus (BVDV) 1a, BVDV1b, BVDV2, bovine herpesvirus-1, parainfluenza-3 virus bovine respiratory syncytial virus, *Mannheimia haemolytica* and *Pasteurella* multocida in beef calves, antibody decline by half-life studies and effect on response to vaccination. *Vaccine* 22, 643–649.

Gagea, M.I., Bateman, K.G., van Dreumel, T., McEwen, B.J., Carman, S., Archambault, M., Shanahan, R.A. and Caswell, J.L. (2006) Diseases and pathogens associated with mortality in Ontario beef feedlots. *Journal of Veterinary Diagnostic Investigation* 18, 18–28.

Galinski, M.S., Troy, R.M. and Banerjee, A.K. (1992) RNA editing in the phosphoprotein gene of the human parainfluenza virus type 3. *Virology* 186, 543–550.

Garcin, D., Itoh, M. and Kolakofsky, D. (1997) A point mutation in the Sendai virus accessory C proteins attenuates virulence for mice, but not virus growth in cell culture. *Virology* 238, 424–431.

Garcin, D., Curran, J., Itoh, M. and Kolakofsky, D. (2001) Longer and shorter forms of Sendai virus C proteins play different roles in modulating the cellular antiviral response. *Journal of Virology* 75, 6800–6807.

Garcin, D., Marq, J.B., Strahle, L., le Mercier, P. and Kolakofsky, D. (2002) All four Sendai virus C proteins bind Stat1, but only the larger forms also induce its mono-ubiquitination and degradation. *Virology* 295, 256–265.

Garten, W., Berk, W., Nagai, Y., Rott, R. and Klenk, H.D. (1980) Mutational changes of the protease susceptibility of glycoprotein F of Newcastle disease virus: effects on pathogenicity. *Journal of General Virology* 50, 135–147.

Gates, G.A., Cesario, T.C., Ebert, J.W., Kriel, R.L., Wulff, H.T., Poland, J.D., Gutekunst, D.E. and Chin, T.D. (1970) Neutralizing antibody in experimentally induced respiratory infection in calves. *American Journal of Veterinary Research* 31, 217–224.

Geijtenbeek, T.B. and Gringhuis, S.I. (2009) Signalling through C-type lectin receptors: shaping immune responses. *Nature Reviews Immunology* 9, 465–479.

Glezen, W.P., Frank, A.L., Taber, L.H. and Kasel, J.A. (1984) Parainfluenza virus type 3: seasonality and risk of infection and reinfection in young children. *Journal of Infectious Diseases* 150, 851–857.

Goodbourn, S. and Randall, R.E. (2009) The regulation of type I interferon production by paramyxoviruses. *Journal of Interferon and Cytokine Research* 29, 539–547.

Gotoh, B., Takeuchi, K., Komatsu, T. and Yokoo, J. (2003) The STAT2 activation process is a crucial target of Sendai virus C protein for the blockade of alpha interferon signaling. *Journal of Virology* 77, 3360–3370.

Grdzelishvili, V.Z., Smallwood, S., Tower, D., Hall, R.L., Hunt, D.M. and Moyer, S.A. (2005) A single amino acid change in the L-polymerase protein of vesicular stomatitis virus completely abolishes viral mRNA cap methylation. *Journal of Virology* 79, 7327–7337.

Griffin, D. (1997) Economic impact associated with respiratory disease in beef cattle. *Veterinary Clinics of North America Food Animal Practice* 13, 367–377.

Hamaguchi, M., Yoshida, T., Nishikawa, K., Naruse, H. and Nagai, Y. (1983) Transcriptive complex of Newcastle disease virus. I. Both L and P proteins are required to constitute an active complex. *Virology* 128, 105–117.

Hamdy, A.H. (1966) Association of myxovirus parainfluenza-3 with pneumoenteritis of calves: virus isolation. *American Journal of Veterinary Research* 27, 981–986.

Hiebert, S.W., Paterson, R.G. and Lamb, R.A. (1985) Hemagglutinin-neuraminidase protein of the paramyxovirus simian virus 5: nucleotide sequence of the mRNA predicts an N-terminal membrane anchor. *Journal of Virology* 54, 1–6.

Hore, D.E. and Stevenson, R.G. (1969) Respiratory infection of lambs with an ovine strain parainfluenza virus type 3. *Research in Veterinary Science* 10, 342–350.

Horikami, S.M., Curran, J., Kolakofsky, D. and Moyer, S.A. (1992) Complexes of Sendai virus NP-P and P-L proteins are required for defective interfering particle genome replication in vitro. *Journal of Virology* 66, 4901–4908.

Horikami, S.M., Smallwood, S. and Moyer, S.A. (1996) The Sendai virus V protein interacts with the NP protein to regulate viral genome RNA replication. *Virology* 222, 383–390.

Horvath, C.M., Paterson, R.G., Shaughnessy, M.A., Wood, R. and Lamb, R.A. (1992) Biological activity of paramyxovirus fusion proteins: factors influencing formation of syncytia. *Journal of Virology* 66, 4564–4569.

Horwood, P.F., Gravel, J.L. and Mahony, T.J. (2008) Identification of two distinct bovine parainfluenza virus type 3 genotypes. *Journal of General Virology* 89, 1643–1648.

Johnson, K. and Morein, B. (1977) In vitro stimulation of bovine circulating lymphocytes by parainfluenza type 3 virus. *Research in Veterinary Science* 22, 83–85.

Karron, R.A. and Collins, P.L. (2007) Parainfluenzaviruses. In: Knipe, D.M. and Howley, P.M. (eds) *Fields Virology*. Lippincott Williams & Wilkins, Philadelphia, Pennsylvania.

Kato, A., Ohnishi, Y., Kohase, M., Saito, S., Tashiro, M. and Nagai, Y. (2001) Y2, the smallest of the Sendai virus C proteins, is fully capable of both counteracting the antiviral action of interferons and inhibiting viral RNA synthesis. *Journal of Virology* 75, 3802–3810.

King, A.M.Q., Adams, M.J., Carstens, E.B. and Lefkowitz, E.J. (eds) (2012) *Virus Taxonomy: Classification and Nomenclature of Viruses: Ninth Report of the International Committee on Taxonomy of Viruses*. Elsevier Academic Press, San Diego, California.

Klenk, H.D. and Garten, W. (1994) Host cell proteases controlling virus pathogenicity. *Trends in Microbiology* 2, 39–43.

Klippmark, E., Rydbeck, R., Shibuta, H. and Norrby, E. (1990) Antigenic variation of human and bovine parainfluenza virus type 3 strains. *Journal of General Virology* 71, 1577–1580.

Kolakofsky, D., Pelet, T., Garcin, D., Hausmann, S., Curran, J. and Roux, L. (1998) Paramyxovirus RNA synthesis and the requirement for hexamer genome length: the rule of six revisited. *Journal of Virology* 72, 891–899.

Kolakofsky, D., Roux, L., Garcin, D. and Ruigrok, R.W. (2005) Paramyxovirus mRNA editing, the 'rule of six' and error catastrophe: a hypothesis. *Journal of General Virology* 86, 1869–1877.

Komatsu, T., Takeuchi, K., Yokoo, J. and Gotoh, B. (2004) C and V proteins of Sendai virus target signaling pathways leading to IRF-3 activation for the negative regulation of interferon-beta production. *Virology* 325, 137–148.

Komatsu, T., Takeuchi, K. and Gotoh, B. (2007) Bovine parainfluenza virus type 3 accessory proteins that suppress beta interferon production. *Microbes and Infection* 9, 954–962.

Lamb, R.A. (1993) Paramyxovirus fusion: a hypothesis for changes. *Virology* 197, 1–11.

Lamb, R.A. and Parks, G.D. (2007) Paramyxoviridae. In: Knipe, D.M. and Howley, P.M. (eds) *Fields Virology*. Lippincott Williams & Wilkins, Philadelphia, Pennsylvania, pp. 1449–1496.

Lamb, R.A., Mahy, B.W. and Choppin, P.W. (1976) The synthesis of sendai virus polypeptides in infected cells. *Virology* 69, 116–131.

Lamb, R.A., Paterson, R.G. and Jardetzky, T.S. (2006) Paramyxovirus membrane fusion: lessons from the F and HN atomic structures. *Virology* 344, 30–37.

Liggitt, D., Huston, L., Silflow, R., Evermann, J. and Trigo, E. (1985) Impaired function of bovine alveolar macrophages infected with parainfluenza-3 virus. *American Journal of Veterinary Research* 46, 1740–1744.

Lin, G.Y., Paterson, R.G., Richardson, C.D. and Lamb, R.A. (1998) The V protein of the paramyxovirus SV5 interacts with damage-specific DNA binding protein. *Virology* 249, 189–200.

Liston, P. and Briedis, D.J. (1994) Measles virus V protein binds zinc. *Virology* 198, 399–404.

Luthra, P., Sun, D., Wolfgang, M. and He, B. (2008) AKT1-dependent activation of NF-kappaB by the L protein of parainfluenza virus 5. *Journal of Virology* 82, 10,887–10,895.

Lyon, M., Leroux, C., Greenland, T., Chastang, J., Patet, J. and Mornex, J.F. (1997) Presence of a unique parainfluenza virus 3 strain identified by RT-PCR in visna-maedi virus infected sheep. *Veterinary Microbiology* 57, 95–104.

MacLachlan, N.J. and Dubovi, E.J. (2011) Paramyxoviridae: bovine parainfluenzavirus 3. In: MacLachlan, N.J. and Dubovi, E.J. (eds) *Fenner's Veterinary Virology*, 4th edn. Academic Press, London, pp. 308–309.

Maidana, S.S., Lomonaco, P.M., Combessies, G., Craig, M.I., Diodati, J., Rodriguez, D., Parreño, V., Zabal, O., Konrad, J.L., Crudelli, G., Mauroy, A., Thiry, E. and Romera, S.A. (2012) Isolation and characterization of bovine parainfluenza virus type 3 from water buffaloes (*Bubalus bubalis*) in Argentina. *Biomed Central Veterinary Research* 8, 83.

Marshall, R.G. and Frank, G.H. (1971) Neutralizing antibody in serum and nasal secretions of calves exposed to parainfluenza-3 virus. *American Journal of Veterinary Research* 32, 1699–1706.

Marshall, R.G. and Frank, G.H. (1975) Clinical and immunologic responses of calves with colostrally acquired maternal antibody against parainfluenza-3 virus to homologous viral infection. *American Journal of Veterinary Research* 36, 1085–1089.

McFerran, J.B. and McNulty, M.S. (1981) Modern diagnostic methods in practice: aids to diagnosis of virological diseases. *British Veterinary Journal* 137, 455–463.

McKercher, D.G., Saito, J.K., Franti, C.E., Wada, E.M. and Crenshaw, G.L. (1972) Response of calves to parainfluenza-3 vaccines administered nasally or parenterally. *American Journal of Veterinary Research* 33, 721–730.

McNeil, J.W., Paschal, J.C., McNeil, M.S. and Morgan, W.W. (1996) Effect of morbidity on performance and profitability of feedlot steers. *Journal of Animal Science* Suppl. 1, 74.

Melchjorsen, J., Jensen, S.B., Malmgaard, L., Rasmussen, S.B., Weber, F., Bowie, A.G., Matikainen, S. and Paludan, S.R. (2005) Activation of innate defense against a paramyxovirus is mediated by RIG-I and TLR7 and TLR8 in a cell-type-specific manner. *Journal of Virology* 79, 12,944–12,951.

Melikyan, G.B., Markosyan, R.M., Roth, M.G. and Cohen, F.S. (2000) A point mutation in the transmembrane domain of the hemagglutinin of influenza virus stabilizes a hemifusion intermediate that can transit to fusion. *Molecular Biology of the Cell* 11, 3765–3775.

Morein, B. (1970) Immunity against parainfluenza-3 virus in cattle. IgA in nasal secretions. *International Archives of Allergy and Applied Immunology* 39, 403–414.

Morein, B. and Moreno-Lopez, J. (1973) Skin hypersensitivity to parainfluenza-3 virus in cattle. *Zentralblatt Für Veterinarmedizin B* 20, 540–546.

Moreno-Lopez, J. (1977) Cell-mediated immunity to parainfluenza-3 (PIV-3) in cattle. Evaluation of in vivo and in vitro tests. *Zentralblatt fur Veterinarmedizin B* 24, 231–240.

Morrison, T.G. (2003) Structure and function of a paramyxovirus fusion protein. *Biochimica et Biophysica Acta* 1614, 73–84.

Moyer, S.A., Baker, S.C. and Lessard, J.L. (1986) Tubulin: a factor necessary for the synthesis of both Sendai virus and vesicular stomatitis virus RNAs. *Proceedings of the National Academy of Science USA* 83, 5405–5409.

Muchmore, H.G., Parkinson, A.J., Humphries, J.E., Scott, E.N., McIntosh, D.A., Scott, L.V., Cooney, M.K. and Miles, J.A. (1981) Persistent parainfluenza virus shedding during isolation at the South Pole. *Nature* 289, 187–189.

Nagai, Y. (1999) Paramyxovirus replication and pathogenesis. Reverse genetics transforms understanding. *Reviews in Medical Virology* 9, 83–99.

Nagai, Y. and Klenk, H.D. (1977) Activation of precursors to both glycoporteins of Newcastle disease virus by proteolytic cleavage. *Virology* 77, 125–134.

Nagai, Y., Klenk, H.D. and Rott, R. (1976) Proteolytic cleavage of the viral glycoproteins and its significance for the virulence of Newcastle disease virus. *Virology* 72, 494–508.

Nichani, A.K., Dar, M.A., Mirakhur, K.K., Krieg, A.M., Booth, J.S., Townsend, H.G., Potter, A.A., Babiuk, L.A. and Mutwiri, G.K. (2010) Subcutaneous, but not intratracheal administration of the TLR9 agonist, CpG DNA transiently reduces parainfluenza-3 virus shedding in newborn lambs. *Comparative Immunology, Microbiology and Infectious Diseases* 33, e111–117.

O'Neill, R.G., Fitzpatrick, J.L., Glass, E.J., Williams, J.L. and Woolliams, J.A. (2007) Optimisation of the response to respiratory virus vaccines in cattle. *Veterinary Record* 161, 269–270.

Ogino, T. and Banerjee, A.K. (2007) Unconventional mechanism of mRNA capping by the RNA-dependent RNA polymerase of vesicular stomatitis virus. *Molecular Cell* 25, 85–97.

Ogino, T., Yamadera, T., Nonaka, T., Imajoh-Ohmi, S. and Mizumoto, K. (2001) Enolase, a cellular glycolytic enzyme, is required for efficient transcription of Sendai virus genome. *Biochemical and Biophysical Research Communications* 285, 447–455.

Ogino, T., Iwama, M., Ohsawa, Y. and Mizumoto, K. (2003) Interaction of cellular tubulin with Sendai virus M protein regulates transcription of viral genome. *Biochemical and Biophysical Research Communications* 311, 283–293.

Ohsawa, K., Yamada, A., Takeuchi, K., Watanabe, Y., Miyata, H. and Sato, H. (1998) Genetic characterization of parainfluenza virus 3 derived from guinea pigs. *Journal of Veterinary Medical Science* 60, 919–922.

Omar, A.R., Jennings, A.R. and Betts, A.O. (1966) The experimental disease produced in calves by the J-121 strain of parainfluenza virus type 3. *Research in Veterinary Science* 7, 379–388.

Palosaari, H., Parisien, J.P., Rodriguez, J.J., Ulane, C.M. and Horvath, C.M. (2003) STAT protein interference and suppression of cytokine signal transduction by measles virus V protein. *Journal of Virology* 77, 7635–7644.

Parks, J.B., Post, G., Thorne, T. and Nash, P. (1972) Parainfluenza-3 virus infection in Rocky Mountain bighorn sheep. *Journal of Americal Veterinary Medical Association* 161, 669–672.

Paterson, R.G. and Lamb, R.A. (1990) RNA editing by G-nucleotide insertion in mumps virus P-gene mRNA transcripts. *Journal of Virology* 64, 4137–4145.

Paterson, R.G., Leser, G.P., Shaughnessy, M.A. and Lamb, R.A. (1995) The paramyxovirus SV5 V protein binds two atoms of zinc and is a structural component of virions. *Virology* 208, 121–131.

Poch, O., Blumberg, B.M., Bougueleret, L. and Tordo, N. (1990) Sequence comparison of five polymerases (L proteins) of unsegmented negative-strand RNA viruses: theoretical assignment of functional domains. *Journal of General Virology* 71, 1153–1162.

Precious, B., Young, D.F., Bermingham, A., Fearns, R., Ryan, M. and Randall, R.E. (1995) Inducible expression of the P, V, and NP genes of the paramyxovirus simian virus 5 in cell lines and an examination of NP-P and NP-V interactions. *Journal of Virology* 69, 8001–8010.

Probert, A.J. and Lwin, T. (1976) Fasciola hepatica: the presence of particle-associated and soluble nonspecific acid phosphatases. *Experimental Parasitology* 40, 206–211.

Qiao, D., Janke, B.H. and Elankumaran, S. (2009) Molecular characterization of glycoprotein genes and phylogenetic analysis of two swine paramyxoviruses isolated from United States. *Virus Genes* 39, 53–65.

Reisinger, R.C., Heddleston, K.L. and Manthei, C.A. (1959) A myxovirus (SF-4) associated with shipping fever of cattle. *Journal of Americal Veterinary Medical Association* 135, 147–152.

Rodriguez, J.J., Wang, L.F. and Horvath, C.M. (2003) Hendra virus V protein inhibits interferon signaling by preventing STAT1 and STAT2 nuclear accumulation. *Journal of Virology* 77, 11,842–11,845.

Rydbeck, R., Love, A., Orvell, C. and Norrby, E. (1987) Antigenic analysis of human and bovine parainfluenza virus type 3 strains with monoclonal antibodies. *Journal of General Virology* 68, 2153–2160.

Sabbah, A. and Bose, S. (2009) Retinoic acid inducible gene I activates innate antiviral response against human parainfluenza virus type 3. *Virology Journal* 6, 200.

Sakaguchi, T., Kiyotani, K., Kato, A., Asakawa, M., Fujii, Y., Nagai, Y. and Yoshida, T. (1997) Phosphorylation of the Sendai virus M protein is not essential for virus replication either *in vitro* or *in vivo*. *Virology* 235, 360–366.

Sanderson, C.M., McQueen, N.L. and Nayak, D.P. (1993) Sendai virus assembly: M protein binds to viral glycoproteins in transit through the secretory pathway. *Journal of Virology* 67, 651–663.

Scheid, A. and Choppin, P.W. (1974) Identification of biological activities of paramyxovirus glycoproteins. Activation of cell fusion, hemolysis, and infectivity of proteolytic cleavage of an inactive precursor protein of Sendai virus. *Virology* 57, 475–490.

Schmidt, A.C., McAuliffe, J.M., Huang, A., Surman, S.R., Bailly, J.E., Elkins, W.R., Collins, P.L., Murphy, B.R. and Skiadopoulos, M.H. (2000) Bovine parainfluenza virus type 3 (BPIV3) fusion and hemagglutinin-neuraminidase glycoproteins make an important contribution to the restricted replication of BPIV3 in primates. *Journal of Virology* 74, 8922–8929.

Schmitt, A.P., He, B. and Lamb, R.A. (1999) Involvement of the cytoplasmic domain of the hemagglutinin-neuraminidase protein in assembly of the paramyxovirus simian virus 5. *Journal of Virology* 73, 8703–8712.

Sergel, T., McGinnes, L.W., Peeples, M.E. and Morrison, T.G. (1993) The attachment function of the Newcastle disease virus hemagglutinin-neuraminidase protein can be separated from fusion promotion by mutation. *Virology* 193, 717–726.

Shaw, M.L., Garcia-Sastre, A., Palese, P. and Basler, C.F. (2004) Nipah virus V and W proteins have a common STAT1-binding domain yet inhibit STAT1 activation from the cytoplasmic and nuclear compartments, respectively. *Journal of Virology* 78, 5633–5641.

Shephard, M.J., Todd, D., Adair, B.M., Po, A.L., Mackie, D.P. and Scott, E.M. (2003) Immunogenicity of bovine parainfluenza type 3 virus proteins encapsulated in nanoparticle vaccines, following intranasal administration to mice. *Research in Veterinary Science* 74, 187–190.

Shibuta, H., Kanda, T., Adachi, A. and Yogo, Y. (1979) Characterization of bovine parainfluenza virus type 3. *Microbiology Immunology* 23, 617–628.

Shibuta, H., Kanda, T., Hazama, A., Adachi, A. and Matumoto, M. (1981) Parainfluenza 3 virus: plaque-type variants lacking neuraminidase activity. *Infection and Immunity* 34, 262–267.

Shibuta, H., Adachi, A., Kanda, T. and Matumoto, M. (1982) Experimental parainfluenzavirus infection in mice: fatal illness with atrophy of thymus and spleen in mice caused by a variant of parainfluenza 3 virus. *Infection and Immunity* 35, 437–441.

Shibuta, H., Kanda, T., Nozawa, A., Sato, S. and Kumanishi, T. (1985) Experimental parainfluenza virus infection in mice: growth and spread of a highly pathogenic variant of parainfluenza 3 virus in the mouse brain. *Archives of Virology* 83, 43–52.

Shibuta, H., Suzu, S. and Shioda, T. (1986) Differences in bovine parainfluenza 3 virus variants studied by monoclonal antibodies against viral glycoproteins. *Virology* 155, 688–696.

Skiadopoulos, M.H., Schmidt, A.C., Riggs, J.M., Surman, S.R., Elkins, W.R., St Claire, M., Collins, P.L. and Murphy, B.R. (2003) Determinants of the host range restriction of replication of bovine parainfluenza virus type 3 in rhesus monkeys are polygenic. *Journal of Virology* 77, 1141–1148.

Slauson, D.O., Lay, J.C., Castleman, W.L. and Neilsen, N.R. (1987) Alveolar macrophage phagocytic kinetics following pulmonary parainfluenza-3 virus infection. *Journal of Leukocyte Biology* 41, 412–420.

Snowder, G.D., Van Vleck, L.D., Cundiff, L.V. and Bennett, G.L. (2006) Bovine respiratory disease in feedlot cattle: environmental, genetic, and economic factors. *Journal of Animal Science* 84, 1999–2008.

Stevenson, R.G. and Hore, D.E. (1970) Comparative pathology of lambs and calves infected with parainfluenza virus type 3. *Journal of Comparative Pathology* 80, 613–618.

Stott, E.J., Probert, M. and Thomas, L.H. (1975) Cytotoxicity of alveolar macrophages for virus-infected cells. *Nature* 255, 710–712.

Stricker, R., Mottet, G. and Roux, L. (1994) The Sendai virus matrix protein appears to be recruited in the cytoplasm by the viral nucleocapsid to function in viral assembly and budding. *Journal of General Virology* 75, 1031–1042.

Takeuchi, O. and Akira, S. (2009) Innate immunity to virus infection. *Immunological Reviews* 227, 75–86.

Takeuchi, K., Komatsu, T., Yokoo, J., Kato, A., Shioda, T., Nagai, Y. and Gotoh, B. (2001) Sendai virus C protein physically associates with STAT1. *Genes Cells* 6, 545–557.

Tarbouriech, N., Curran, J., Ruigrok, R.W. and Burmeister, W.P. (2000a) Tetrameric coiled coil domain of Sendai virus phosphoprotein. *Nature Structural Biology* 7, 777–781.

Tarbouriech, N., Curran, J., Ebel, C., Ruigrok, R.W. and Burmeister, W.P. (2000b) On the domain structure and the polymerization state of the sendai virus P protein. *Virology* 266, 99–109.

Thomas, L.H. and Stott, E.J. (1975) Comparison of three methods for sampling the bovine upper respiratory tract for viruses. *Research in Veterinary Science* 18, 227–229.

Thomas, S.M., Lamb, R.A. and Paterson, R.G. (1988) Two mRNAs that differ by two nontemplated nucleotides encode the amino coterminal proteins P and V of the paramyxovirus SV5. *Cell* 54, 891–902.

Thompson, P.N., Stone, A. and Schultheiss, W.A. (2006) Use of treatment records and lung lesion scoring to estimate the effect of respiratory disease on growth during early and late finishing periods in South African feedlot cattle. *Journal of Animal Science* 84, 488–498.

Thompson, S.D., Laver, W.G., Murti, K.G. and Portner, A. (1988) Isolation of a biologically active soluble form of the hemagglutinin-neuraminidase protein of Sendai virus. *Journal of Virology* 62, 4653–4660.

Thorsen, J. and Henderson, J.P. (1971) Survey for antibody to infectious bovine rhinotracheitis (IBR), bovine virus diarrhea (BVD) and parainfluenza 3 (PI3) in moose sera. *Journal of Wildlife Diseases* 7, 93–95.

Tsai, K.S. and Thomson, R.G. (1975) Bovine parainfluenza type 3 virus infection: ultrastructural aspects of viral pathogenesis in the bovine respiratory tract. *Infection and Immunity* 11, 783–803.

Unterholzner, L., Keating, S.E., Baran, M., Horan, K.A., Jensen, S.B., Sharma, S., Sirois, C.M., Jin, T., Latz, E., Xiao, T.S., Fitzgerald, K.A., Paludan, S.R. and Bowie, A.G. (2010) IFI16 is an innate immune sensor for intracellular DNA. *Nature Immunology* 11, 997–1004.

USDA-APHIS (2001) Treatment of respiratory disease in US feedlots. Info sheet APHIS Veterinary Services (http://www.aphis.usda.gov/animal_health/animal_dis_spec/downloads/eradication_status.pdf).

Van der Maaten, M.J. (1969) Immunofluorescent studies of bovine para-influenza 3 virus. I. Cell cultures and experimentally infected hamsters. *Canadian Journal of Comparative Medicine* 33, 134–140.

Vangeel, I., Ioannou, F., Riegler, L., Salt, J.S. and Harmeyer, S.S. (2009) Efficacy of an intranasal modified live bovine respiratory syncytial virus and temperature-sensitive parainfluenza type 3 virus vaccine in 3-week-old calves experimentally challenged with PI3V. *Veterinary Journal* 179, 101–108.

Whelan, S.P. (2008) Response to 'Non-segmented negative-strand RNA virus RNA synthesis *in vivo*'. *Virology* 371, 234–237.

Windeyer, M.C., Leslie, K.E., Godden, S.M., Hodgins, D.C., Lissemore, K.D. and Leblanc, S.J. (2012) The effects of viral vaccination of dairy heifer calves on the incidence of respiratory disease, mortality, and growth. *Journal of Dairy Science* 95, 6731–6739.

Wittum, T.E., Woollen, N.E., Perino, L.J. and Littledike, E.T. (1996) Relationships among treatment for respiratory tract disease, pulmonary lesions evident at slaughter, and rate of weight gain in feedlot cattle. *Journal of American Veterinary Medical Association* 209, 814–818.

Woods, G.T., Sibinovic, K. and Marquis, G. (1965) Experimental exposure of calves, lambs, and colostrum-deprived pigs to bovine myxovirus parainfluenza-3. *American Journal of Veterinary Research* 26, 52–56.

Woods, G.T., Mansfield, M.E., Cmarik, G., Zinzilieta, M. and Marquis, G. (1968) Vaccination of beef calves before weaning with a live-virus bovine myxovirus parainfluenza-3 vaccine. *American Journal of Veterinary Research* 29, 1349–1353.

Yanagihara, R. and McIntosh, K. (1980) Secretory immunological response in infants and children to parainfluenza virus types 1 and 2. *Infection and Immunity* 30, 23–28.

Yener, Z., Saglam, Y.S., Timurkaan, N. and Ilhan, F. (2005) Immunohistochemical detection of parainfluenza type 3 virus antigens in paraffin sections of pneumonic caprine lungs. *Journal of Veterinary Medicine A: Physiology, Pathology, Clinical Medicine* 52, 268–271.

Zaitsev, V., von Itzstein, M., Groves, D., Kiefel, M., Takimoto, T., Portner, A. and Taylor, G. (2004) Second sialic acid binding site in Newcastle disease virus hemagglutinin-neuraminidase: implications for fusion. *Journal of Virology* 78, 3733–3741.

Zarnke, R.L. and Erickson, G.A. (1990) Serum antibody prevalence of parainfluenza 3 virus in a free-ranging bison (*Bison bison*) herd from Alaska. *Journal of Wildlife Diseases* 26, 416–419.

Zhao, H. and Banerjee, A.K. (1995) Interaction between the nucleocapsid protein and the phosphoprotein of human parainfluenza virus 3. Mapping of the interacting domains using a two-hybrid system. *Journal of Biological Chemistry* 270, 12,485–12,490.

Zhu, Y.M., Shi, H.F., Gao, Y.R., Xin, J.Q., Liu, N.H., Xiang, W.H., Ren, X.G., Feng, J.K., Zhao, L.P. and Xue, F. (2011) Isolation and genetic characterization of bovine parainfluenza virus type 3 from cattle in China. *Veterinary Microbiology* 149, 446–451.

8 Swine Parainfluenza Virus 3

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8.1 History and Importance

Paramyxoviruses infect a large and diverse range of host species, including avian, porcine, canine, bovine, equine, ovine, reptile, aquatic species and humans (Franke *et al*., 2001; Lamb and Parks, 2007; Horwood *et al*., 2008; Nollens *et al*., 2008; Nylund *et al*., 2008). In 1979, Mapuera virus (MPRV) was isolated from an apparently healthy tropical fruit bat (*Sturnira lilium*) in Brazil (Karabatsos, 1985). MPRV was closely related genetically to La Piedad Michoacan paramyxovirus (LPMV) also known as porcine rubulavirus (PoRV), the only well-studied neurotropic paramyxovirus isolated from pigs prior to the 1990s. The host range and disease-causing potential of MPRV is still unknown. LPMV was first isolated in central Mexico in the early 1980s (Moreno-Lopez *et al*., 1986), and it has become endemic in Mexico (Linne *et al*., 1992). This virus induced interstitial pneumonia and encephalitis in pigs. There were extensive records of paramyxoviruses derived from the brain or nasal swabs of sick pigs in Japan in the 1950s (Philbey *et al*., 1998), Canada in 1971 (Ellis *et al*., 1998), in Israel in 1986 (Janke *et al*., 2001), as well as in the USA in the 1960s and in Texas the 1980s (Janke *et al*., 2001). There was also concurrent infection of porcine reproductive and respiratory syndrome virus and a paramyxovirus in Germany in the 1990s (Heinen *et al*., 1998) that has been subsequently named 'SER' virus (Tong *et al*., 2002).

A paramyxovirus named 'Texas 81' was isolated from the brain of pigs that exhibited respiratory and neurological disease in Texas state in 1981 (cited in Janke *et al*., 2001). Further information on the history of this virus is not available. The first record of the outbreak of a swine paramyxovirus named 'ISU 92' was described by Janke *et al*. (2001). A group of 400 pigs in a continuous-flow finishing barn had signs of respiratory and CNS disease. The complete genome of Texas 81 and ISU 92 viruses has been characterized and their pathogenicity determined. It is proposed to designate them as 'Swine parainfluenza virus 3' (SPIV3) (Qiao *et al*., 2009, 2010).

Since 1994, four bat-associated paramyxoviruses have emerged, three of which caused disease in animals and humans (Chua *et al*., 2001; Wang *et al*., 2001). In late 1994 in Australia, Hendra virus (HeV) caused an outbreak of severe respiratory disease resulting in the death of 13 horses and their trainer (Murray *et al*., 1995), followed by sporadic HeV outbreaks in horses and humans (Field *et al*., 2007). A closely related virus, Nipah virus (NiV), from Malaysia caused severe febrile encephalitis

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and death in pigs and humans (Chua *et al.*, 2000). It then spread to Bangladesh and India (Hsu *et al.*, 2004; Chadha *et al.*, 2006). In 1997, another paramyxovirus, named Menangle virus (MenV), was isolated in Australia from still-born pigs with deformities (Philbey *et al.*, 1998), and associated human illness (Chant *et al.*, 1998). In 2000, Tioman virus (TioV) was isolated from urine collected beneath a fruit bat colony on Tioman Island, Malaysia (Chua *et al.*, 2001). Molecular characterization revealed that MenV and TioV are closely related novel members of the genus Rubulavirus (Chua *et al.*, 2001, 2002; Bowden and Boyle, 2005). J-virus (JV), isolated from wild mice in Australia, and Beilong virus (BeV), originally isolated from human mesangial cells in China and subsequently detected in rat mesangial cells, represent a new group of paramyxoviruses (Jun *et al.*, 1977; Basler *et al.*, 2005; Jack *et al.*, 2005; Li *et al.*, 2006). Recently, novel paramyxoviruses were also isolated from Atlantic bottlenose dolphins and Atlantic salmon and characterized (Nollens *et al.*, 2008; Nylund *et al.*, 2008).

For many of the new and emerging viruses, the host range, pathogenicity and geographic distribution are unknown. The recent surge in isolations of paramyxoviruses from a wide host range suggests the flexibility of the virus to adapt in different hosts. Continued surveillance and characterization of these viruses are important to effectively contain outbreaks induced by them.

8.2 Epizootiology

The distribution SPIV3 in pigs and other species is unknown. An earlier seroprevalence study with 876 serum samples collected from 36 swine farms in Iowa between 1988 and 1989 revealed that only six samples, representing five swine herds, had anti-ISU92 antibodies by a serum neutralization test (Battrell, 1995). Another study examining the prevalence of bovine parainfluenza virus 3 (BPIV3) antibodies in 1392 pig sera from 195 farms in Minnesota in 1988 by a haemagglutination inhibition test indicated that only 12.4% of sera tested positive at dilutions of 1:40 and above (Tehteh and Goyal, 1988). In a recent seroprevalence study, 100 serum samples from pigs aged 19–70 days collected from five swine farms in 2007–2008 were negative by SPIV3-specific ELISA (Qiao *et al.*, 2010), suggesting that SPIV3 is not circulating among pigs in the USA at present.

8.3 Infectious Agent

8.3.1 Classification

SPIV3 is an enveloped, non-segmented, negative sense, single-stranded RNA virus with genomic and morphologic features characteristic of members of the genus Respirovirus in the Paramyxoviridae family, order Mononegavirales (Qiao *et al.*, 2009). The other members of the genus Respirovirus include human parainfluenza viruses 1 and 3 (HPIV1 and HPIV3), BPIV3 and Sendai virus (Karron and Collins, 2007). The virions of PIV are morphologically indistinguishable and also share distinct biophysical characteristics. All of them possess haemagglutinating and neuraminidase activities (Karron and Collins, 2007). There are three genotypes of BPIV3, designated A, B and C. Previously, complete genome analyses of the representative BPIV3 isolates from Australia and North America indicated that BPIV3 falls into two distinct genotypes, BPIV3 genotype A (BPIV3a) and BPIV3 genotype B (BPIV3b) (Horwood *et al.*, 2008). Recently, four Chinese isolates of BPIV3 were proposed as genotype C (Zhu *et al.*, 2011). The SPIV3 isolates are genetically very closely related to BPIV3a (Qiao *et al.*, 2010).

8.3.2 Virion structure

SPIV3 virions are spherical to pleomorphic, approximately 50–300 nm in diameter and morphologically indistinguishable from other paramyxoviruses (Qiao *et al.*, 2009).

Intact virions are enveloped and densely packed with surface projections representing viral glycoprotein spikes. Nucleocapsids are visible and exhibit a typical 'herringbone' pattern (Fig. 8.1).

8.3.3 Genome features

The complete genome of Texas 81 is 15,456 nucleotides (nt) in length and that of ISU 92 is 15,480 nt. The genome length is divisible by six and consistent with the 'rule of six' as described for most other members of the Paramyxoviridae (Calain and Roux, 1993; Kolakofsky *et al.*, 1998, 2005). The genome contains six discrete, non-overlapping transcription units. The coding capacity of the genome is 93.3% in Texas 81 and 93.2% in ISU 92. Both viruses have conserved gene start (GS), gene end (GE) and strictly conserved trinucleotide intergenic sequence (IGS), for all the six genes compared with BPIV3 (Qiao *et al.*, 2010). ISU 92 shares identical GS and GE with BPIV3-910N strain, while Texas 81 shares identical GS and GE with BPIV3-SF/Ka strain, except the GS of L gene, which is identical with BPIV3-910N strain.

The genomic termini of members of the Paramyxoviridae are observed to have complementarity between the 3′ and 5′ termini (Li *et al.*, 2005). These conserved terminal sequences, especially the first 12~13 nt, are believed to contain the genome and antigenome promoters essential for replication

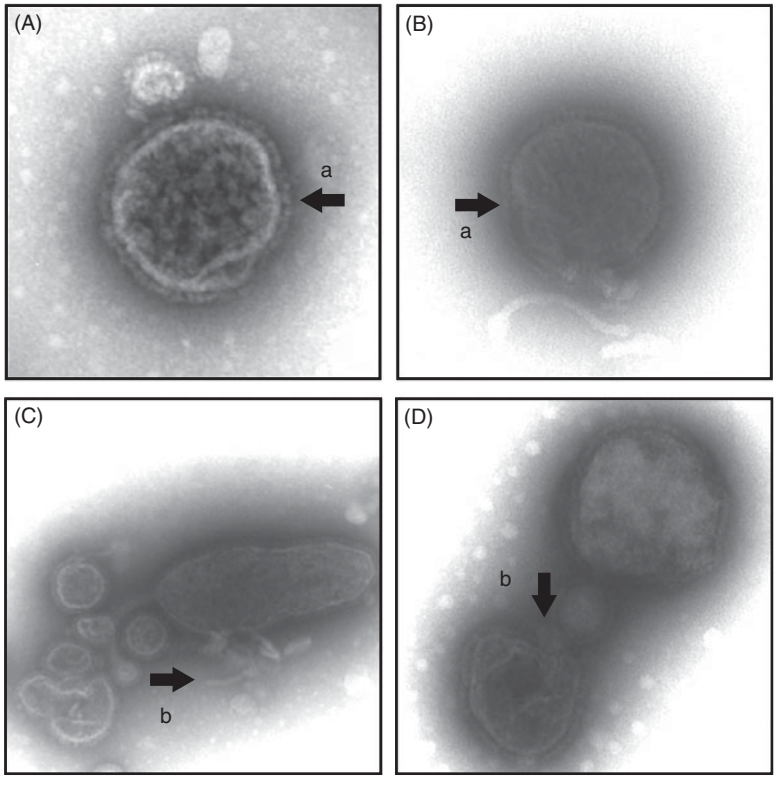


Fig. 8.1. Transmission electronmicrographs of swine paramyxoviruses. Purified virions were negatively stained with 1% phosphotungstic acid and viewed under a transmission electron microscope. (A, C) ISU 92 virus, (B, D) Texas 81 virus. Intact virion with fine surface projections (arrow a) representing the viral glycoprotein spikes. Highly pleomorphic viral particles with extruded nucleocapsids (arrow b). Nucleocapsids exhibit a typical 'herringbone' pattern.

and transcription (Lamb and Parks, 2007). The swine viruses have a 55 nt 3′ leader before the transcription start site for the N gene, the length of which is conserved among almost all of the members of the Paramyxovirinae subfamily. The 5′ trailer of swine viruses is 44-nt long following the L gene transcription stop site and the length is variable among the Paramyxovirinae subfamily, but conserved among parainfluenza type 3 (PIV3) viruses. Overall, the swine viruses possess a 96.4% (53 nt out of 55 nt) identity in the leader region with BPIV3, and a 92.7% (51 nt out of 55 nt) identity with HPIV3. In the trailer region there is a 97.7% (43 nt out of 44 nt) identity between swine viruses and BPIV3, and 90.9% (40 nt out of 44 nt) identity with HPIV3. The exact complementarity at the first 14 nt of the 3′ genomic leader and overall 65.9% (ISU 92)/63.6% (Texas 81) complementarity (29(ISU 92)/28 (Texas 81) nt out of 44 nt) between the 3′ leader and 5′ trailer termini suggest conserved elements in the 3′ promoter regions of the genome and antigenome (Fig. 8.2).

8.3.4 Genome organization

The genome organization of swine viruses can best be described as 3′-N-P/V/D-M-F-HN-L-5′ and can potentially encode nine

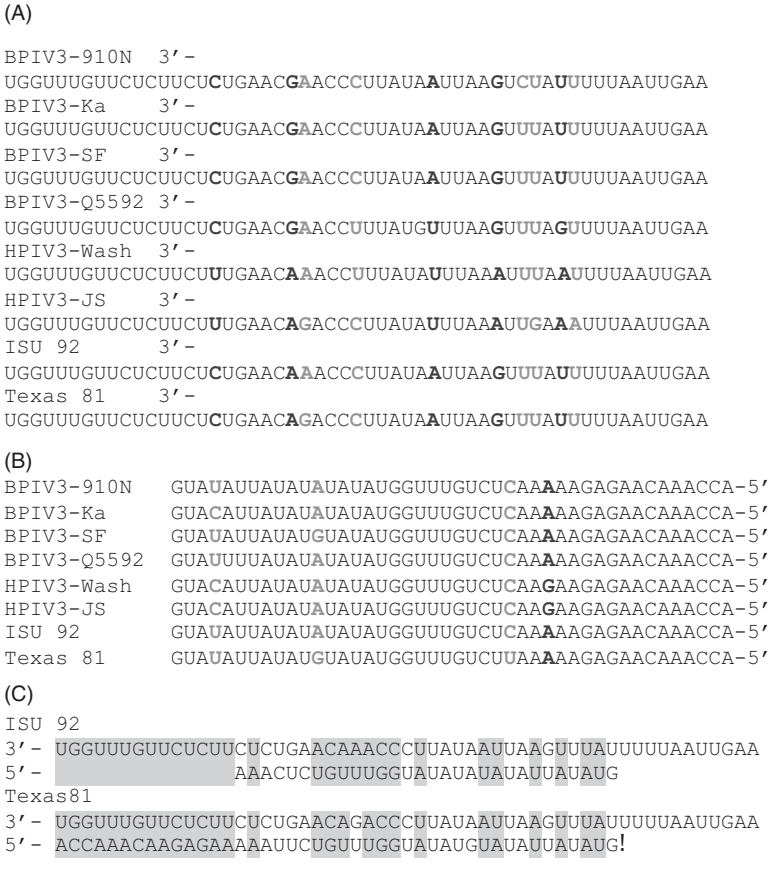


Fig. 8.2. Nucleotide sequence of the 3′ leader (A), 5′ trailer (B) regions and genomic termini (C) of SPIV3 genomic RNA. Sequences are 3′ to 5′ in negative sense. (A, B) Nucleotide residues that are host-specific (BPIV3 or HPIV3) are made bold. Host-specific positions are those where BPIV3 strains share an assignment and HPIV3 strains share a different assignment. Positions that display variability within a host species are in bold and light colour. (C) Shading indicates complementary base pairs.

proteins. By inserting a broad distribution of Gs at the mRNA editing site, V and D proteins are predicted to express from the P gene of both ISU 92 and Texas 81. The features of six genes and corresponding proteins of two viruses are shown in Table 8.1. The identity of Texas 81 with ISU 92 genome is 94.1% at the nucleotide level. The identity between swine viruses and BPIV3 is 98.2% at the highest (between Texas 81 and BPIV3-SF) level, while the identity between swine viruses and HPIV3 is 80.1% at the highest (between Texas 81 and HPIV3-JS) level. Analyses of the start sites of each gene and the P gene editing site of Texas 81 and ISU 92, and the hexamer phasing positions of '2,1,1,1,1,2,2', which is shown to be genus-specific within the Paramyxovirinae (Kolakofsky *et al.*, 1998; Harcourt *et al.*, 2001) are identical with HPIV3 and BPIV3 (Table 8.2).

8.4 Viral Proteins

The fusion protein (F) and an attachment protein called haemagglutinin-neuraminidase (HN), haemagglutinin (H) or glycoprotein (G) are the major components of the virion envelope. The F and HN proteins are responsible for mediating virus attachment and penetration during infection. The internal helical nucleocapsid core contains the RNA genome and nucleocapsid (N), phospho- (P) and large (L) polymerase proteins. Another structural protein, matrix (M) protein, is located between

Table 8.1. Comparison of gene sequences between swine, bovine and human PIV3 viruses.

		mRNA				Deduced protein		
Gene	Virus	Length	3′ UTR (nt)	ORF (nt)	5′ UTR (nt)	Length	Calculated MW	pI
N	Texas 81	1646	55	1545	46	515	57,303.70	5.009
	ISU 92	1646	55	1545	46	515	57,340.89	5.217
	BPIV3[a]	1646	55	1545	46	515	57,303.70	5.009
	HPIV3	1646	55	1545	46	515	57,840.25	5.212
P	Texas 81	1995	79	1788	128	596	69,185.44	5.789
	ISU 92	1995	79	1788	128	596	66,290.29	5.718
	BPIV3	1995	79	1788	128	596	66,459.46	5.576
	HPIV3	2013	79	1809	125	603	67,794.65	5.292
M	Texas 81	1149	32	1053	64	351	39,327.09	9.473
	ISU 92	1149	32	1053	64	351	39,252.06	9.610
	BPIV3	1149	32	1053	64	351	39,327.09	9.473
	HPIV3	1155	32	1059	64	353	39,571.39	9.582
F	Texas 81	1869	211	1620	38	540	60,188.74	6.539
	ISU 92	1893	235	1620	38	540	60,039.42	6.797
	BPIV3	1869	211	1620	38	540	60,188.74	6.539
	Texas 81	1893 (910N)	235		38		60,007.41	7.025
		1887 (Q5592)	223		44		60,128.69	6.796
	ISU 92	1851	193	1617	41	539	59,978.35	7.499
HN	BPIV3	1888	73	1716	99	572	64,624.47	7.720
	HPIV3	1888	73	1716	99	572	64,652.46	7.343
	Texas 81	1888	73	1716	99	572	64,623.48	7.869
	ISU 92	1912 (Q5592)	97				64,431.41	7.504
	BPIV3	1888	73	1716	99	572	64,313.66	7.546
L	HPIV3	6795	22	6699	74	2233	255,734.70	6.281
	Texas 81	6795	22	6699	74	2233	255,814.76	6.161
	ISU 92	6795	22	6699	74	2233	255,801.85	6.220
	BPIV3	6795	22	6699	74	2233	256,071.48	6.394

[a]BPIV3 indicates BPIV3 shipping fever stain if not specified. HPIV3: human parainfluenzavirus 3.

Table 8.2. Subunit hexamer phasing positions for gene start sites and P editing sites of a selection of Paramyxovirinae.

Genus	Virus(es)	N	P/V	M	F	HN	L	P Edit
Respirovirus	SPIV3	2	1	1	1	1	2	2
	HPIV3, BPIV3	2	1	1	1	1	2	2
	SeV	2	1	1	1	1	2	1
Rubulavirus	SV5	2	1	1	2	1	6	3
	MuV	2	1	6	1	1	6	3
Morbillivirus	CDV, DMV	2	2	4	2	3	2	6
	MeV, RPV	2	2	4	3	3	2	6
Avulavirus	NDV	2	4	4	4	3	6	1
Henipavirus	HeV	2	3	4	4	4	3	5
Unclassified	ASPV	2	2	1	2	3	4	2
	TPMV	2	2	1	3	3	2	6
	FDLV	2	2	4	3	6	3	2

ASPV: Atlantic Salmon Paramyxovirus; CDV: canine distemper virus; DMV: Dolphin morbillivirus; FDLV: Fer-de-Lance virus; HeV: hendravirus; HPIV3: human parainfluenzavirus 3; MeV: measles virus; MuV: mumps virus; NDV: Newcastle disease virus; RPV: rinderpest virus; SeV: Sendai virus; SV5: Simian virus 5; SPIV3: swine parainfluenzavirus 3; TPMV: Tupaia paramyxovirus.

the envelope and the core, and is important in virion architecture and the infection process. Accessory proteins are mostly generated from overlapping open reading frames (ORF) within P gene transcriptional units by a process called RNA editing or by alternative translation initiation. These accessory proteins are important in viral morphogenesis, RNA synthesis and pathogenesis (Lamb and Parks, 2007).

8.4.1 Nucleoprotein (N)

The N gene of both Texas 81 and ISU 92 is 1646-nt long and encodes a N protein of 515 amino acid (aa) long, with a predicted molecular weight (MW) of 57.3 kDa (Yener *et al.*, 2005) and isoelectric point (pI) of 5.0 (Texas 81) and 5.2 (ISU 92), respectively. The N protein tightly binds to the entire length of genomic and antigenomic RNA to form the nucleocapsid and it is also associated with the polymerase complex during transcription and replication. A highly conserved motif located near the middle of all members of Paramyxovirinae N protein, which is thought to be essential in N–N self-assembly and the N–RNA interaction process, is F-X4-Y-X3-Ø-S-Ø-A-M (where X is any residue and Ø is an aromatic amino acid) (Myers *et al.*, 1997; Lamb and Parks, 2007). This motif is also seen within the central domain of the N protein of swine viruses, presented as ^{323}FAPGNYPALWSYAM^{336}. In SeV and other paramyxoviruses, the first residue of this motif F324 (annotated in SeV) is needed for correct self-assembly and another residue Y260 (annotated in SeV) (Myers *et al.*, 1997) is critical for N–viral RNA binding. These two residues are conserved in swine viruses as F323 and Y259, respectively. The last 24% sequence of carboxy terminal of N (aa 394 to 515) has a very low identity with other Paramyxoviridae members. This region with consistently low similarity is where most of the phosphorylation and antigenic sites of the protein (Karron and Collins, 2007; Lamb and Parks, 2007) are located. The N protein of ISU 92 and Texas 81 has 97.1% amino acid sequence identity with each other. When aligned with selected Paramyxoviridae members, the identities decreased in order: subfamily Paramyxovirinae, Respirovirus (60.6–100%); Morbillivirus (18.9–23.4%); Henipavirus (19.8–20.2%); Avulavirus (17.7–20.6%); Rubulavirus (17.4–19.2%); subfamily Pneumovirinae, Metapneumovirus (11.5–12.2%); Pneumovirus (11.1–11.8%); unclassified viruses, ASPV (23.4–24.8%), BeV (23.3–23.8%), J-V (20.2–21.5%) (Table 8.3).

8.4.2 The phosphoprotein (P) and RNA editing

The P gene of the two strains of SPIV3 is 1995-nt long with a major ORF of 1788 nt encoding the large P protein of 596-aa long with a calculated size of 66.3 kDa (ISU 92) and 69.2 kDa (Texas 81) and pI of 5.4 and 5.6, respectively. The sequence identity between two SPIV3 P genes is 87.6% and

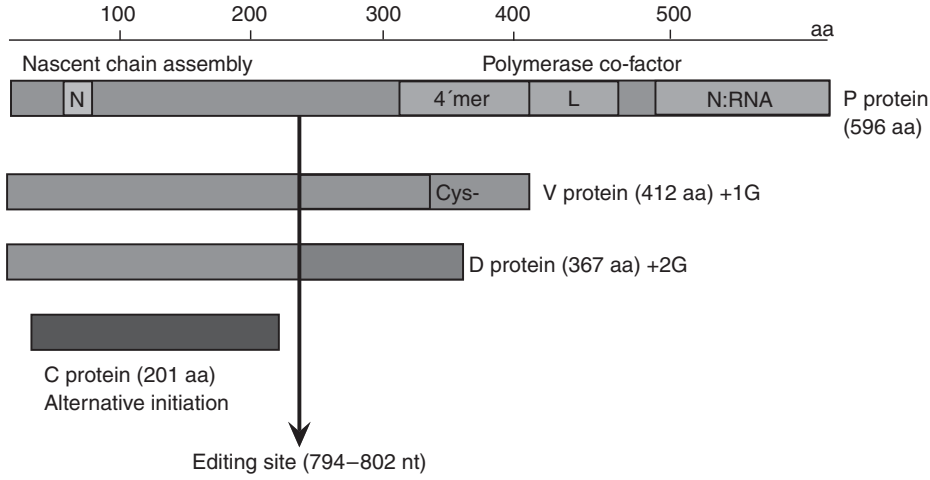
Table 8.3. Amino acid identities of swine parainfluenzaviruses with analogous proteins in other paramyxoviruses.

		% Amino acid identity between SPIV3 and other paramyxovirus proteins											
		N		P		M		F		A		L	
Paramyxovirus genus	Species	T	I	T	I	T	I	T	I	T	I	T	I
Respirovirus	SPIV3	97.1		87.6		96.6		95.0		96.0		99.2	
	BPIV3	100	97.1	100	87.6	100	96.6	100	95.0	99.8	95.8	98.7	98.2
	HPIV3	86.2	86.4	64.3	65.4	92.6	92.0	82.6	81.9	77.1	77.6	90.6	90.6
	HPIV1	60.6	61.0	24.1	24.5	64.6	63.1	42.7	42.7	45.8	45.6	60.7	60.7
	SeV	62.6	62.6	23.7	24.5	64.0	63.7	43.1	42.9	46.7	46.0	59.7	59.7
Rubulavirus	MenV	19.2	19.2	9.2	9.2	19.9	19.9	23.4	23.0	15.2	15.4	28.5	28.5
	SV5	18.5	18.8	8.8	8.5	18.4	18.1	24.8	24.0	25.9	25.5	29.4	29.4
	HPIV2	18.5	18.6	6.4	5.9	18.7	18.4	25.2	25.0	24.0	24.4	28.4	28.3
	LPMV	18.3	18.8	8.5	8.8	18.2	18.8	24.4	24.4	25.6	25.6	28.5	28.4
Avulavirus	NDV	20.1	20.3	5.9	5.9	19.9	20.5	25.0	24.9	23.2	23.0	26.2	26.3
	APMV2	20.6	20.0	10.7	10.2	17.9	18.2	22.0	22.2	21.0	21.2	27.2	27.1
	APMV6	17.9	17.7	7.6	8.8	22.9	22.3	24.2	24.6	20.9	20.5	26.8	26.8
Morbillivirus	MeV	20.8	20.8	9.4	9.4	37.2	37.2	25.7	25.7	11.6	11.8	37.6	37.6
	RPV	19.8	19.4	9.5	9.7	37.2	37.2	24.8	24.2	11.6	11.3	37.4	37.6
Henipavirus	NiV	20.2	19.8	10.0	10.0	32.5	31.9	27.7	28.1	18.9	19.4	39.7	39.7
	HeV	20.2	19.8	10.6	10.0	32.7	32.5	26.9	26.7	18.9	19.6	38.8	38.8
Metapneumovirus	HMPV	12.2	11.5	13.9	13.9	10.6	9.8	15.3	15.3	10.6	11.0	16.7	16.8
	AMPV	12.0	11.5	14.3	15.0	9.8	9.1	14.6	14.8	8.8	9.1	17.0	17.0
Pneumovirus	HRSV	11.1	11.5	12.0	12.0	9.4	9.4	13.2	12.9	11.7	12.3	15.1	15.1
	BRSV	11.1	11.8	12.9	14.0	9.8	9.8	12.9	13.2	12.0	12.0	15.4	15.1
Unclassified virus	ASPV	24.8	23.4	9.5	10.5	39.2	40.1	31.7	31.7	37.0	37.4	48.3	48.5
	BeV	23.8	23.3	8.9	9.1	36.0	35.7	28.0	27.6	24.2	24.4	37.9	38.0
	J-V	20.5	21.5	7.5	7.8	36.0	35.7	28.9	29.3	24.1	24.8	38.6	38.8
	FDLV	21.4	21.7	11.3	12.3	36.0	35.8	30.1	30.3	36.4	36.6	42.4	42.5

A: attachment protein. The attachment protein for morbilliviruses is H; for henipaviruses, metapneumoviruses, pneumoviruses, BeV, J-V is G; for remaining is HN. T: Texas 81; I: ISU 92; the row SPIV3 indicates the identities between the two swine strains. AMPV: avian metapneumovirus; APMV: avian paramyxovirus; ASPV: Atlantic Salmon Paramyxovirus; BeV: Beilaong virus; BPIV3: bovine parainfluenzavirus 3; BRSV: bovine respiratory syncytial virus; FDLV: Fer-de-Lance virus; HeV: hendravirus; HMPV: human metapneumonia virus; HPIV: human parainfluenzavirus; HRSV: human respiratory syncytial virus; J-V: J virus; LPMV: La Piedad Michoagan paramyxovirus; MenV: Menangle virus; MeV: measles virus; NDV: Newcastle disease virus; NiV: Nipah virus; RPV: rinderpest virus; SeV: Sendai virus; SPIV3: swine parainfluenzavirus 3; SV5: Simian virus 5.

when compared with other Paramyxovirus P proteins it is poorly conserved (Table 8.3). The N terminals of P protein of paramyxoviruses are heavily phosphorylated at serine and threonine residues (Lamb and Parks, 2007). There are 44 serine and 16 threonine residues in Texas 81 P protein and 50 serine and 17 threonine residues in ISU 92 P protein identified as potential phosphorylation sites (Qiao *et al.*, 2010).

The P gene contains an mRNA editing site, 5′AAAAAAGGG3′ (mRNA sense) in both Texas 81 and ISU 92 (794–802 nt), which is identical to those of other Respiroviruses (Fig. 8.3). Texas 81 has 1–4 G insertions, while ISU 92 had a broader distribution of G insertions (1–9 Gs) at the P gene editing sites (Qiao *et al.*, 2010). The insertion of G residues during mRNA synthesis can shift the translational reading frame and thus potentially generate V (+1/4/7 Gs) protein, predicted to be 412-aa long of 47.8 kDa in ISU 92 and 46.7 kDa in Texas 81, with identity of 83.3% between two strains, and D (+2/5/8 Gs) protein, 367-aa long of 41.3 kDa in ISU 92 and 41.5 kDa in Texas 81 with identity of 84.0% between the two strains. The predicted V and D proteins will be amino co-terminal with P (+0/3/6/9 Gs) protein (first 241 aa). In SPIV3, the C protein is predicted to be initiated 10-nt downstream of the P protein start



sPIV3	AAAAAA	GGG
Respirovirus	AAAAAA	GGG
Morbillivirus	AAAAA	GGG
Rubulavirus	AAGA	GGGG(n)
Avulavirus	AAAAA	GGG
Henipavirus	AAAAA	GGG
ASPV	AAAAAA	GG
J-V	AAAAAA	GG
BeV	AAAAA	GG
FDLV	AUAA	GGGGGG
TPMV	AAAAAA	GG

Fig. 8.3. Schematic of P protein structure, P gene expression strategy and RNA editing site sequence of swine viruses compared with other paramyxoviruses. The scale bar at the top indicates the amino acid of P protein, and the boxes below indicate possible reading frames of protein P, V, D and C. Known functional domains are indicated for chaperoning unassembled N proteins during the nascent chain assembly of genome replication (N), self-assembly as a tetramer (4′mer), L protein-binding site (L) and N-RNA-binding site (N:RNA) based on the study of Sendai virus (Lamb, 2007). In the editing site sequences, A_nG_n elements are spacing between A_n and G_n to facilitate visual comparison (Hausmann *et al.*, 1999; Lamb, 2007).

codon by alternate translation initiation AUG codon and predicted to be 201-aa long with MW of 23.6 kDa in ISU 92 and 23.7 kDa in Texas 81, with identity of 91.1% between the two strains (Qiao *et al.*, 2010).

At the C-terminal of V protein, SPIV3 contains all seven conserved invariantly placed cysteine residues, highly conserved motifs H-R-R-E and W-C-N-P, known among paramyxovirus V proteins (Qiao *et al.*, 2010). This cysteine-rich C-terminal is shown to be involved in coordinating two zinc molecules per V protein and may play an important role in viral pathogenesis and blocking of host interferon defence mechanisms (Patterson *et al.*, 2000; He *et al.*, 2002; Poole *et al.*, 2002; Lamb and Parks, 2007).

8.4.3 Matrix protein (M)

The M gene is 1149-nt long with a single ORF of 1053 nt (Qiao *et al.*, 2010). The encoded protein is 351-aa long with a predicted MW of 39.3 kDa and a pI of 9.5 (Texas 81) and 9.6 (ISU 92). The M protein is the most abundant virion structural protein located in the inner surface of envelope. It interacts with the cytoplasmic tails of the integral membrane proteins, lipid bilayer and the nucleocapsids, and plays an important role in virion assembly, budding, release as well as transport of viral components (Karron and Collins, 2007; Lamb and Parks, 2007). M protein is considered to be the most conserved parainfluenza viral protein (Spriggs *et al.*, 1987). The nuclear localization signal (NLS) (^{245}KMGRMYSVEYCKQKIEK^{261}) of M protein (Peeples *et al.*, 1992; Coleman and Peeples, 1993) in swine viruses is highly conserved (Qiao *et al.*, 2010). The ISU 92 virus M protein has 96.6% amino acid sequence identity with Texas 81. The amino acid sequence identity with members of the other genera of Paramyxovirinae decreased in the following order: Respirovirus (63.1–100%); Morbillivirus (33.5–37.2%); Henipavirus (31.9–32.7%); Avulavirus (17.9–22.9%); Rubulavirus (18.1–19.9%); with unclassified viruses, ASPV (39.2–40.1%), J-V (35.7–36%), BeV (35.7–36%). There was only less than 10% identity when SPIV3 were compared with the members of Pneumovirinae (Qiao *et al.*, 2010).

8.4.4 Fusion protein (F)

The GS and GE sequences of the F and HN genes are essentially similar to BPIV3. Only the variable fifth and sixth positions of the GS sequence showed host-specificity (Qiao *et al.*, 2009). All of them terminated with U-rich GE sequences. The trinucleotide IGS (3′-GAA) of these two viruses are also identical to members of the genera Respirovirus, Morbillivirus and Henipavirus (Bailly *et al.*, 2000; Qiao *et al.*, 2009). A long U-rich sequence was identified in the 3′ UTR of F gene of ISU 92, but not in Texas 81. This is a characteristic feature commonly found in BPIV3 and HPIV3. This has been shown to result in read-through transcripts of M gene in BPIV3 and HPIV3 (Sakai *et al.*, 1987; Suzu *et al.*, 1987).

The F gene of Texas 81 strain is 1869-nt in length with a single ORF of 1620 nt beginning at position 211 (Table 8.1), capable of encoding a 540-aa protein. The F gene of ISU 92 strain is 1893 nt in length with a 1620 nt ORF but a longer 3′ UTR region (235 nt). The long 3′ UTR in ISU 92 strain has a 24 nt 'U' rich insertion compared with the Texas 81 strain. The F protein of ISU 92 has a predicted molecular weight of 60,039 Da and an estimated pI of 6.797. For Texas 81 strain, the uncleaved F_0 protein has a predicted molecular weight of 60,189 Da and an estimated pI of 6.539. The Texas 81 strain has 100% identity with BPIV3-SF strain both in nucleotide and deduced amino acid sequences. ISU 92 strain has 98.7% identity in nucleotide sequence and 99.1% identity in amino acid sequences with BPIV3-SK217 strain, while it has 90.9% and 95% identity with BPIV3-SF strain in nucleotide and amino acid sequences, respectively (Table 8.3) for F gene (Qiao *et al.*, 2009).

The F protein mediates fusion of virus and cell membrane in paramyxoviruses.

Fusion activation is dependent on the cleavage of F_0 protein into disulfide-linked subunits F_2-s-s-F_1. In Texas 81 and ISU 92 viruses, the F cleavage motif L/SRTKR is located between amino acid residues 105 to 109, and cleavage occurs between residues 109 (R) and 110 (F). This cleavage site conforms to the pattern of consensus motif for cleavage by furin (Hosaka *et al.*, 1991), R-X-K/R-R, which is conserved in the majority of Paramyxovirinae (Lamb and Parks, 2007). The predicted F cleavage site is immediately followed by a 25-aa hydrophobic fusion peptide, highly conserved in all paramyxovirus F proteins (Horvath *et al.*, 1992; Lamb and Parks, 2007). The cleaved F_1 protein is approximately 51 kDa in size. The F protein of both strains, like the F protein of other paramyxoviruses, is predicted to be a type I membrane protein (Fig. 8.4). The transmembrane (Ortmann *et al.*, 1994) region is near the carboxyl terminal (amino acid residues 497–517), which is considered to serve as an anchor in the viral envelope, leaving a 23-aa cytoplasmic tail (Qiao *et al.*, 2009).

The F proteins of swine paramyxoviruses are essentially similar in structure and function to other paramyxoviruses, and especially to BPIV3. Peptides corresponding to the heptad repeat regions A and B (HRA and HRB) from SV5 (Baker *et al.*, 1999), HRSV (Zhao *et al.*, 2000) and HPIV3 (Yin *et al.*, 2005), are conserved in the F protein of SPIV3. The F proteins are assembled into stable six helical bundles (6HBs) (the F_1 core), and their structure in HPIV3 has been determined. Structure-based sequence alignment of the SPIV3 F protein sequences with these 6HB fragments revealed only minor differences. The conserved blocks in F_1 and F_2 (CBF_1, CBF_2) are also preserved in SPIV3 as in other paramyxoviruses, which strengthens the hypothesis that these conserved blocks have conserved functions, either in the membrane fusion or in the folding and processing of the F protein (Gardner and Dutch, 2007). Cysteines, which are important for disulfide bond formation and secondary structure, are also identical to BPIV3 and HPIV3. Among the

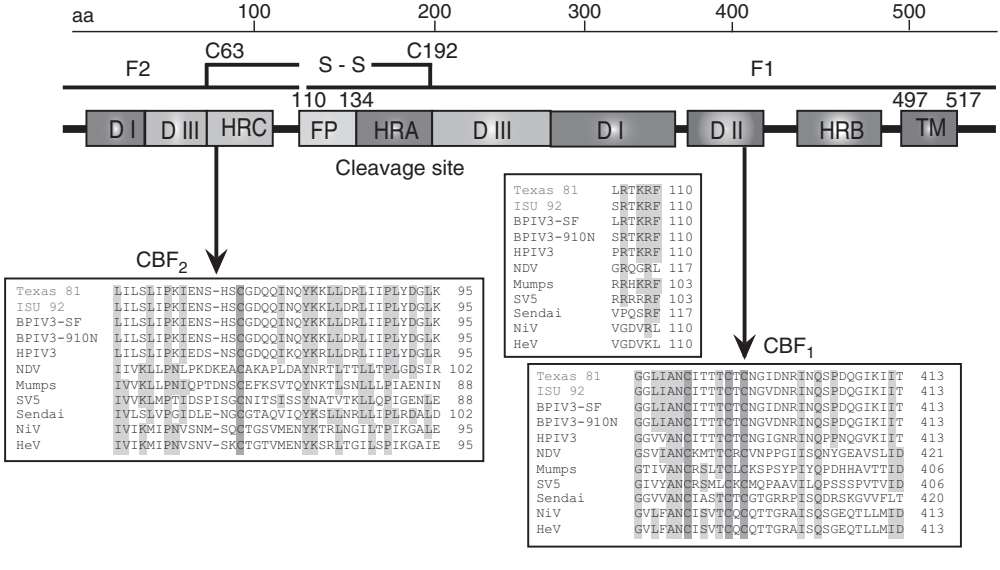


Fig. 8.4. Schematic of the predicted domain structure of the SPIV3 F_0 protein and identification of conserved block in F1 (CBF_1), conserved block in F2 (CBF_2) and fusion peptide (FP) regions were obtained from structure-based sequence alignment of the F protein of SPIV3 with other paramyxoviruses. Domains are indicated as DI to DIII. TM indicates transmembrane domain. HRA, HRB and HRC indicate heptad repeat regions. Conserved amino acid residues are shaded. BPIV3: bovine parainfluenzavirus 3; HeV: Hendra virus; HPIV3: human parainfluenzavirus 3; NDV: Newcastle disease virus; NiV: Nipah virus; SV5: Simian virus 5.

five potential conserved N-linked glycosylation sites (N101, N238, N359, N446 and N508) in the F protein (Suzu *et al.*, 1987), all but one has the N-X-T motif. The one at position 446 has the N-X-S motif. It should be noted that only one potential glycosylation site (at N101) is located before the F protein cleavage site, and one site is located in the TM domain (N508). The other three are located prior to the TM domain in the F_1 protein and are, therefore, likely to be exposed on the surface, as in other members of the genus Respirovirus (Qiao *et al.*, 2009).

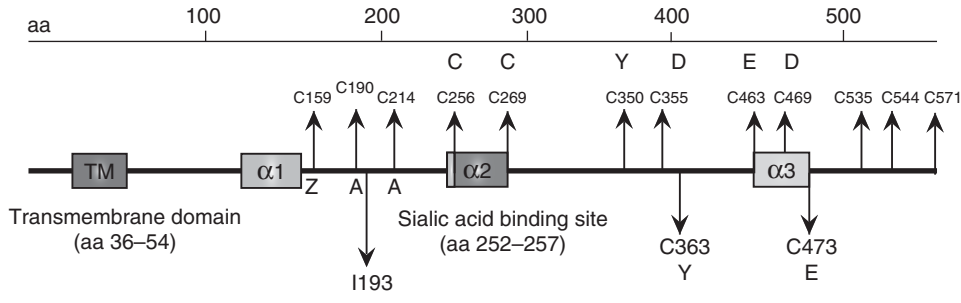
8.4.5 Haemagglutination-neuraminidase protein (HN)

The HN gene is 1888 nt in length with a single ORF beginning at position 74 that could encode a 572-aa protein (Table 8.1). Texas 81 strain shows 99.8% identity with BPIV3-SF strain, both in nucleotide and predicted amino acid sequences. ISU 92 strain shows 98.4% nucleotide sequence identity and 99.1% amino acid sequence identity with BPIV3-SK217 strain. Identity of ISU 92 with BPIV3-SF strain is 91.7% at nucleotide level and 95.8% at amino acid level (Table 8.3). Compared with HPIV3, the two swine viruses have 80.1~82.7% identities in the nucleotide sequences and 76.4~77.6% identity at deduced amino acid sequences. The deduced amino acid sequence of the HN protein is 572 residues in length. The HN protein of ISU 92 has a predicted molecular weight of 64,652 Da and an estimated pI of 7.343. The HN protein of Texas 81 strain has a predicted molecular weight of 64,624 Da and an estimated pI of 7.720 (Qiao *et al.*, 2009). The active site residues predicted from NDV or HPIV3 HN crystal structure are also conserved in the swine viruses. The major TM region of HN protein is predicted to be from amino acid residues 36–54 of the protein, as in other type II membrane glycoproteins. The TM domain at the N-terminal end of the HN protein contained several conserved substitutions among the examined viruses (Fig. 8.5).

The disulfide bonds in the HN protein C190–C214 (A), C256–C269 (C), C355–C469 (D), C463–C473 (E), C535–C544 (F), C159–C571 (W) and C350–C363 (X) are conserved as in NDV HN or HPIV3 HN crystal structures (Crennell *et al.*, 2000; Lawrence *et al.*, 2004). Predicted N-glycosylation sites are conserved in HN proteins as in other members of Paramyxovirinae. In swine viruses, potential N-linked glycan sites observed at all predicted sites (N8, N308, N351, N448 and N523) are similar to HPIV3. The counterpart to N351 in NDV HN is N341, which is glycosylated in that molecule. NDV HN contains a N-linked glycosylation site at N481, but in HPIV3 HN the sequence at this site is N-P-T and in swine viruses it is N-P-S, the asparagine moiety of which is thus not expected to be glycosylated. Texas 81 strain has an additional (N15) N-linked glycosylation acceptor site in the HN protein, which appears to be host specific. Whether all of the potential N-linked glycan sites are glycosylated awaits future studies. Both Texas 81 and ISU 92 viruses have the conserved NRKSCS neuraminidase active site motif (Jorgensen *et al.*, 1987). To date, all analysed members of Respirovirus and Rubulavirus have this sequence (Langedijk *et al.*, 1997).

The sequence analysis shows that Texas 81 and ISU 92 have higher levels of identity with BPIV3 than HPIV1 or HPIV3. The 100% identity at nucleotide level between the F protein of Texas 81 and the SF strain of BPIV3 indicated that cross species transmission might occur among different hosts. On the other hand, ISU 92, which is closely related to the SK217 and 910N strains of BPIV3, possessed host-specific amino acid residues in the F and HN proteins differing from both BPIV3 strains. There are three host specific amino acid residues (Y100, A437 and V501) in the F protein and three in the HN protein (K70, S88 and S387) of ISU 92 strain, compared with BPIV3 and HPIV3. BPIV3 strains SK217 and SF/Ka were shown to differ in virulence (Shibuta *et al.*, 1981; Breker-Klassen *et al.*, 1996). This virulence difference had been associated with a change in amino acid 193 in HN protein, which had a dramatic effect on syncytium-inducing activity, neuraminidase activity and haemagglutinating activity (Breker-Klassen *et al.*, 1996). ISU 92 has an I at this position, unlike SK217 or 910N,

(A)



(B)

	Conserved active site residues			
	HPIV3	NDV	SV5	SPIV3
R1	192	174	163	192
R4	424	416	405	424
R5	503	498	495	503
Y6	530	526	523	530
E4	409	401	390	409
E6	549	547	544	549
D1	216	198	187	216

Fig. 8.5. Schematic of the HN protein globular head region domain structure of swine paramyxovirus. (A) The transmembrane domain, sialic acid site (NRKSCS motif) and the predicted alpha helix regions of SPIV3 are given as shaded boxes. Disulfide bonds followed those used in a structure-based sequence alignment and the crystal structure of Newcastle disease virus (NDV) HN protein (Crennell *et al.,* 2000). (B) The corresponding position of the active site residues in the deduced amino acid sequence of the SPIV3 and other paramyxoviruses (SV5: Simian virus 5).

but identical with Texas 81 and BPIV3-SF/Ka. Further, ISU 92 strain is more fusogenic than the Texas 81 strain. The importance of these residues in host specificity awaits future reverse genetic studies. The molecular features of F and HN genes of SPIV3 are shown in Table 8.3. There are several amino acid changes in the antigenic sites of the F protein differing from HPIV3 but conserved as in BPIV3. They include: E101N, V/T367I (only in ISU 92), S418Q, T492A, T513V (Qiao *et al.*, 2010).

8.4.6 Large polymerase protein (L)

The L gene of SPIV3 is 6795-nt long, with a major 6699-nt long ORF encoding a 2233 aa protein with a pI of 6.3 for Texas 81 or 6.2 for ISU 92, and MW of 25.6 kDa (Qiao *et al.*, 2010). The L protein of PIVs is the major RNA polymerase component and it is responsible for nucleotide polymerization, mRNA capping, methylation and polyadenylation (Lamb and Parks, 2007). It is proposed that there are six highly conserved domains (DI–DVI) in NSV and each domain may be individually responsible for each of the L protein multiple functions (Poch *et al.*, 1990; Sidhu *et al.*, 1993; Svenda *et al.*, 1997). Pairwise alignment of SPIV3 along with the other paramyxoviruses revealed all the six domains, highly conserved A to D subdomains within DIII and a highly variable hinge region between DII and DIII (Fig. 8.6). A highly conserved stretch (positions 543 to 562) within DII, proposed to be a template recognition site, was present in SPIV3 L protein and matched the pattern of basic and hydrophobic amino acid pair repeats at every four residues as described

(Poch *et al.*, 1990). The highly conserved ^{772}GDNQ^{775} motif, which is believed to be the active site for nucleotide polymerization (Malur *et al.*, 2002; Chattopadhyay and Shaila, 2004), was present in subdomain C in DIII. The L protein of swine viruses also contained a putative ATP binding site with motif ^{1786}K-X21-G-E-G-A-G^{1810} (Poch *et al.*, 1990) in DVI. The L protein of Texas 81 has 99.2% amino acid sequence identity with ISU 92, 59.7–98.7% with Respirovirus, 38.8–39.7% with Henipavirus, 37.2–42.5% with Morbillivirus, 28.3–29.8% with Rubulavirus, 26.2–27.2% with Avulavirus, 48.3–48.5% with ASPV, 38.6–38.8% with J-V, 37.9–38% with BeV, 16.7–17.0% with Metapneumovirus and 15.1–15.5% with Pneumovirus.

8.5 Transcription/Replication

There are excellent recent reviews on the complex mechanisms of transcription and replication in NSV (Karron and Collins, 2007; Lamb and Parks, 2007; Banerjee, 2008; Whelan, 2008; Chattopadhyay *et al.*, 2011). The general transcription and replication strategies of SPIV3 are likely to follow other NSVs.

8.6 Phylogenetic Analysis

Phylogenetic trees based on N, P, M, F, HN and L protein sequences and complete genome sequences of SPIV3 and other representative members of all five genera of the family Paramyxoviridae were found to be similar (Qiao *et al.*, 2009, 2010). The SPIV3 strains are phylogenetically closely related to the genus Respirovirus within the Paramyxovirinae subfamily. The phylogenetic reconstructions based on the complete M protein amino acid sequences revealed two distinct genetic groupings formed by BPIV3-910N-like and BPIV3-SF-like viruses. In addition, phylogenetic reconstruction of deduced amino-acid sequences of M proteins and the F and HN

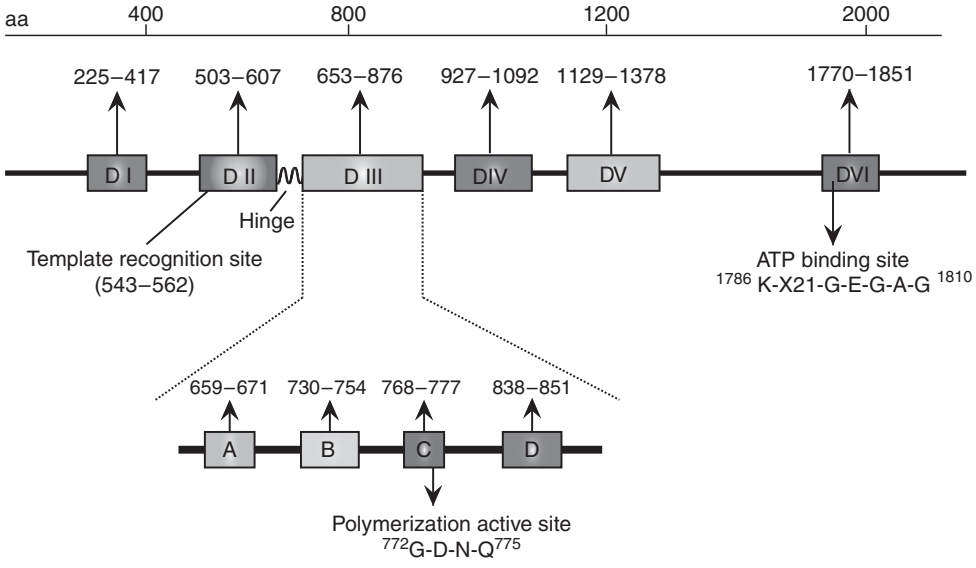


Fig. 8.6. Schematic overview of important features and conserved domains found in the predicted L-protein amino acid sequences. Positions of the conserved RNA-dependent RNA polymerase major domains DI–DVI, and subdomains A–D within DIII are indicated. In addition, the template recognition site in DII, conserved GDNQ sequence in subdomain C and a putative ATP-binding site motif (K–X21–G–E–G–A–G) in major domain VI are shown.

proteins indicate that Texas 81 and ISU 92 belong to two subgenotypes of genotype A of BPIV3 (Qiao *et al.*, 2009, 2010). A recent study claims that Texas 81 and ISU 82 strains have a mosaic L gene with BPIV3-SF and BPIV3-910N, respectively (Yang *et al.*, 2011). Similarity and boot-scanning analysis of Texas 81 revealed four breakpoints located at positions 8688, 12595, 13619 and 14175. The Texas 81 strain demonstrated greater sequence identity and bootscanning support with BPIV3-910N in the second and fourth regions, while otherwise with BPIV3-Q5592 in complementary regions. Neighbour joining analysis indicated that the second and fourth part mosaics had a higher level of congruence with the BPIV3-910N lineage, while otherwise in congruence with BPIV3-SF. In ISU 92, two breakpoints were identified and located at positions 14137 and 14989. The ISU 92 strain demonstrated greater sequence identity and bootscanning support with BPIV3-SF in the middle region while otherwise with BPIV3-Q5592 in the complementary regions. Neighbour joining analysis revealed that pre- and post-part of mosaics had higher level of congruence with the BPIV3-910N lineage, while the mid-part converged with BPIV3-SF (Yang *et al.*, 2011). It is unknown at present whether these recombination events resulted in the host switch of BPIV3 to pigs. It is tempting to speculate this because polymerase proteins are essential for the transcription and replication functions of NSV.

8.7 Antigenic Analysis

Antigenic analysis at the National Veterinary Services Laboratory, Ames, Iowa indicated that SPIV3 were closely related to human parainfluenza virus (HPIV) type 1 and 3 and BPIV3 (Table 8.1). Cross-reactivity in several epitopes in F and HN proteins with HPIV3 and BPIV3 was previously reported (Coelingh *et al.*, 1986). Bovine anti-BPIV3 serum was able to detect the cells infected by these viruses by immunofluorescence (Qiao *et al.*, 2009). As well as using bovine anti-BPIV3 serum, all the viral proteins were detectable in OptiPrep purified virion preparations. Homologous sera could neutralize virus infectivity to similar titres and heterologous serum (BPIV3-SF) neutralized virus infectivity of Texas 81 at 1:32 and ISU 92 at 1:8, suggesting antigenic variation. The *R* values of Texas 81 and ISU 92 strains with BPIV3-SF strain were 100% and 35%, respectively, by immunofluorescence assay (Qiao *et al.*, 2009). These observations suggest that Texas 81 and ISU 92 were antigenically closely related to BPIV3. However, antigenic analysis by immunofluorescence also suggested that ISU 92 is a minor antigenic subtype of BPIV3, according to the criteria of Brooksby (1967).

8.8 Clinical Signs and Pathology

The clinical signs in natural infection with strain ISU 92 pointed towards respiratory and neurological disease (Janke *et al.*, 2001). In the outbreak with SPIV3 ISU 92 strain in a 400-pig continuous-flow finishing barn, sick pigs showed mild cough with temperature 103–104.5°F. Injectable dexamethasone and tylosin were administered and 17 pigs died within 4 days after the first appearance of clinical signs. Continued treatment with a combination of penicillin, dexamethasone, spectinomycin and atropine were used and no more deaths occurred. The disease continued to spread throughout the operation and infected the second finishing all-in/all-out unit. All 400 pigs in the finishing barn were infected within a few days. Severe dyspnoea, coughing and CNS disturbance were observed. Several sick pigs showed persistent squealing, head pressing, whole-body tremors and hind-limb ataxia (Janke *et al.*, 2001). In the breeding, farrowing, nursery and grower buildings, located a quarter of a mile north of the finishing barns, nursery age pigs showed dyspnoea and less cough than finishing pigs; clinical signs in nursing piglets in the farrowing barn were more severe; the grower buildings were only mildly affected; few sows and gilts became dyspnoeic, two or three

abortions and two premature farrowings occurred, however, the cause was not investigated. Injectable antimicrobials were administered to all affected pigs immediately after the first onset of clinical signs and there were no more deaths due to the outbreak (Janke *et al.*, 2001). Affected pigs showed cranio-ventral consolidation of lungs, and microscopically, lungs had moderate to severe bronchointerstitial pneumonia and the brain had mild but widespread lymphocytic perivasculitis and mild diffuse gliosis (Janke *et al.*, 2001).

Intranasal (IN) infection of 10 ml of 20% lung homogenates from a field case into two conventionally reared pigs did not result in any clinical signs or gross/microscopic lesions in infected pigs, but both pigs had seroconverted against cell culture grown ISU 92 strain (Janke *et al.*, 2001). In experimental infection with SPIV3 strain ISU 92 1×10^5 mean tissue culture infective dose ($TCID_{50}$) per ml (4 ml per animal), IN, 3-day old germ-free pigs became rough haired, and developed mild diarrhoea on days 2–3 post-infection (PI) with slightly elevated rectal temperatures (103.6–104.01°F) in 2 out of 4 pigs. Pigs inoculated with virus were not as vigorous as control pigs, but they retained their appetite throughout the trial. Increased respiratory effort and eyelid oedema were noticed on day 2. Less severe gross and histological lesions were noticed in the lungs and brain. The virus was re-isolated from the lungs of infected pigs on day 4 PI. Infected pigs also seroconverted to the ISU 92 strain (Janke *et al.*, 2001). In a recent study with both strains of SPIV3, conventionally reared pigs infected IN with 2 ml (5×10^7 $TCID_{50}$/ml) of virus stock with 1 ml in each nostril showed mild respiratory signs only (Qiao *et al.*, 2010). None of the infected pigs developed neurological signs or elevated body temperatures throughout the experimental period. Two out of 18 pigs from each of the infected groups developed mild respiratory signs at 2 days post inoculation (DPI). One pig from Texas 81 group developed diarrhoea on day 2 and another on day 3. The gross lesions at necropsy on 6 and 10 DPI from virus-infected pigs were unremarkable, such as segmented, thickened small intestine and greenish milky intestinal contents. Histologically, no microscopic lesions were observed. Therefore, it is tempting to generalize SPIV3 as a primary and mild respiratory pathogen similar to BPIV3 (Qiao *et al.*, 2010). Infected pigs had virus-neutralizing antibodies (1:8 to 1:16) at 10 but not at 6 days PI, while an indirect ELISA developed against SPIV3 was able to detect anti-SPIV3 antibodies as early as day 6 PI (Qiao *et al.*, 2010).

8.9 Diagnosis

It is difficult to diagnose SPIV-3 infection in an outbreak of respiratory/diarrhoeal disease in pigs based on clinical signs. The confirmatory diagnosis of SPIV-3 therefore should be made by isolation and identification of the virus and demonstration of increasing antibody titres using paired sera. The most commonly employed serological tests include haemagglutination-inhibition and virus neutralization. An indirect ELISA technique has recently been developed for detecting antibodies against SPIV3 (Qiao *et al.*, 2010). Similar to BPIV3, nasal discharges (Thomas and Stott, 1975) should serve as better samples for virus isolation, but also for rapid immunohistochemical diagnosis. Samples must be collected early in the course of the disease from a number of animals in the herd showing a range of clinical signs. From necropsied animals, fresh or frozen lung tissues harvested from the cranio-ventral areas of lung with lesions are ideal specimens for virus isolation, and antigen detection tests. Reverse-transcriptase polymerase chain reaction (RT-PCR) and quantitative real-time RT-PCR techniques may be employed on nasal swabs, nasal mucus and lung tissues. Because of the multifactorial aetiology of respiratory disease in pigs, the detection of virus, viral antigens or viral nucleic acid does not prove disease causation. Therefore, the test results should be interpreted after taking into consideration the overall clinical condition of the herd and the individual animal.

8.10 Prophylaxis and Control

There are no vaccines available at present for SPIV3 strains. But with the available evidence there is no mounting pressure to develop a vaccine because the prevalence of SPIV3 in the domestic pig population is minimal to absent.

8.11 Conclusions

The genomic organization, amino acid identities of homologous proteins, phylogenetic analysis based on the genome sequences and antigenic analysis all support the classification of these two novel strains of SPIV3 into the Respirovirus genus in the Paramyxovirinae subfamily and Paramyxoviridae family. The mild pathogenicity of SPIV3 can facilitate its development as a vaccine vector. The bovine/HPIV3 chimeric virus (van Wyke Coelingh *et al.*, 1988; Bailly *et al.*, 2000), as a vector backbone with the replacement of F and HN glycoprotein from HPIV3, has been evaluated as a vaccine against HPIV3 (Haller *et al.*, 2000; Pennathur *et al.*, 2003). In addition, BPIV3 as backbone with replaced F and HN of HPIV3 and inserted F of RSV were also constructed. The effectiveness of this vaccine against both PIV3 and RSV challenge has been demonstrated in African green monkeys and is being evaluated in clinical trials (Sato and Wright, 2008). The promising outcome of the vectored vaccines using paramyxoviruses and the biological and molecular characteristics of swine viruses described here suggest a great potential for SPIV3 to be developed as a vaccine vector.

References

Bailly, J.E., McAuliffe, J.M., Skiadopoulos, M.H., Collins, P.L. and Murphy, B.R. (2000) Sequence determination and molecular analysis of two strains of bovine parainfluenza virus type 3 that are attenuated for primates. *Virus Genes* 20, 173–182.

Baker, K.A., Dutch, R.E., Lamb, R.A. and Jardetzky, T.S. (1999) Structural basis for paramyxovirus-mediated membrane fusion. *Molecular Cell* 3, 309–319.

Banerjee, A.K. (2008) Response to 'Non-segmented negative-strand RNA virus RNA synthesis in vivo'. *Virology* 371, 231–233.

Basler, C.F., Garcia-Sastre, A. and Palese, P. (2005) A novel paramyxovirus? *Emerging Infectious Disease* 11, 108–112.

Battrell, M.A. (1995) Cultivation, preliminary characterization, and prevalence in swine herds of porcine paramyxovirus. MS thesis, Iowa State University, Ames.

Bowden, T.R. and Boyle, D.B. (2005) Completion of the full-length genome sequence of Menangle virus: characterisation of the polymerase gene and genomic 5′ trailer region. *Archives of Virology* 150, 2125–2137.

Breker-Klassen, M.M., Yoo, D. and Babiuk, L.A. (1996) Comparisons of the F and HN gene sequences of different strains of bovine parainfluenza virus type 3: relationship to phenotype and pathogenicity. *Canadian Journal of Veterinary Research* 60, 228–236.

Brooksby, J.B. (1967) Variants and immunity: definitions for serological investigation. In: *International Symposium on Foot-and-Mouth Disease: Variants and Immunity*, Lyon, France, 1–10.

Calain, P. and Roux, L. (1993) The rule of six, a basic feature for efficient replication of Sendai virus defective interfering RNA. *Journal of Virology* 67, 4822–4830.

Chadha, M.S., Comer, J.A., Lowe, L., Rota, P.A., Rollin, P.E., Bellini, W.J., Ksiazek, T.G. and Mishra, A. (2006) Nipah virus-associated encephalitis outbreak, Siliguri, India. *Emerging Infectious Disease* 12, 235–240.

Chant, K., Chan, R., Smith, M., Dwyer, D.E. and Kirkland, P. (1998) Probable human infection with a newly described virus in the family Paramyxoviridae. The NSW Expert Group. *Emerging Infectious Disease* 4, 273–275.

Chattopadhyay, A. and Shaila, M.S. (2004) Rinderpest virus RNA polymerase subunits: mapping of mutual interacting domains on the large protein L and phosphoprotein p. *Virus Genes* 28, 169–178.

Chattopadhyay, S., Esper, F. and Banerjee, A.K. (2011) Parainfluenzaviruses, In: Samal, S.K. (ed.) *The Biology of Paramyxoviruses*. Caister Academic Press, Norfolk, UK, pp. 175–209.

Chua, K.B., Bellini, W.J., Rota, P.A., Harcourt, B.H., Tamin, A., Lam, S.K., Ksiazek, T.G., Rollin, P.E., Zaki, S.R., Shieh, W., Goldsmith, C.S., Gubler, D.J., Roehrig, J.T., Eaton, B., Gould, A.R., Olson, J., Field, H., Daniels, P., Ling, A.E., Peters, C.J., Anderson, L.J. and Mahy, B.W. (2000) Nipah virus: a recently emergent deadly paramyxovirus. *Science* 288, 1432–1435.

Chua, K.B., Wang, L.F., Lam, S.K., Crameri, G., Yu, M., Wise, T., Boyle, D., Hyatt, A.D. and Eaton, B.T. (2001) Tioman virus, a novel paramyxovirus isolated from fruit bats in Malaysia. *Virology* 283, 215–229.

Chua, K.B., Wang, L.F., Lam, S.K. and Eaton, B.T. (2002) Full length genome sequence of Tioman virus, a novel paramyxovirus in the genus Rubulavirus isolated from fruit bats in Malaysia. *Archives of Virology* 147, 1323–1348.

Coelingh, K.J., Winter, C.C., Murphy, B.R., Rice, J.M., Kimball, P.C., Olmsted, R.A. and Collins, P.L. (1986) Conserved epitopes on the hemagglutinin-neuraminidase proteins of human and bovine parainfluenza type 3 viruses: nucleotide sequence analysis of variants selected with monoclonal antibodies. *Journal of Virology* 60, 90–96.

Coleman, N.A. and Peeples, M.E. (1993) The matrix protein of Newcastle disease virus localizes to the nucleus via a bipartite nuclear localization signal. *Virology* 195, 596–607.

Crennell, S., Takimoto, T., Portner, A. and Taylor, G. (2000) Crystal structure of the multifunctional paramyxovirus hemagglutinin-neuraminidase. *Nature Structural Biology* 7, 1068–1074.

Ellis, J., Hassard, L., Clark, E., Harding, J., Allan, G., Willson, P., Strokappe, J., Martin, K., McNeilly, F., Meehan, B., Todd, D. and Haines, D. (1998) Isolation of circovirus from lesions of pigs with postweaning multisystemic wasting syndrome. *Canadian Veterinary Journal* 39, 44–51.

Field, H.E., Breed, A.C., Shield, J., Hedlefs, R.M., Pittard, K., Pott, B. and Summers, P.M. (2007) Epidemiological perspectives on Hendra virus infection in horses and flying foxes. *Australian Veterinary Journal* 85, 268–270.

Franke, J., Essbauer, S., Ahne, W. and Blahak, S. (2001) Identification and molecular characterization of 18 paramyxoviruses isolated from snakes. *Virus Research* 80, 67–74.

Gardner, A.E. and Dutch, R.E. (2007) A conserved region in the F(2) subunit of paramyxovirus fusion proteins is involved in fusion regulation. *Journal of Virology* 81, 8303–8314.

Haller, A.A., Miller, T., Mitiku, M. and Coelingh, K. (2000) Expression of the surface glycoproteins of human parainfluenza virus type 3 by bovine parainfluenza virus type 3, a novel attenuated virus vaccine vector. *Journal of Virology* 74, 11,626–11,635.

Harcourt, B.H., Tamin, A., Halpin, K., Ksiazek, T.G., Rollin, P.E., Bellini, W.J. and Rota, P.A. (2001) Molecular characterization of the polymerase gene and genomic termini of Nipah virus. *Virology* 287, 192–201.

He, B., Paterson, R.G., Stock, N., Durbin, J.E., Durbin, R.K., Goodbourn, S., Randall, R.E. and Lamb, R.A. (2002) Recovery of paramyxovirus simian virus 5 with a V protein lacking the conserved cysteine-rich domain: the multifunctional V protein blocks both interferon-beta induction and interferon signaling. *Virology* 303, 15–32.

Heinen, E., Herbst, W. and Schmeer, N. (1998) Isolation of a cytopathogenic virus from a case of porcine reproductive and respiratory syndrome (PRRS) and its characterization as parainfluenza virus type 2. *Archives of Virology* 143, 2233–2239.

Horvath, C.M., Paterson, R.G., Shaughnessy, M.A., Wood, R. and Lamb, R.A. (1992) Biological activity of paramyxovirus fusion proteins: factors influencing formation of syncytia. *Journal of Virology* 66, 4564–4569.

Horwood, P.F., Gravel, J.L. and Mahony, T.J. (2008) Identification of two distinct bovine parainfluenza virus type 3 genotypes. *Journal of General Virology* 89, 1643–1648.

Hosaka, M., Nagahama, M., Kim, W.S., Watanabe, T., Hatsuzawa, K., Ikemizu, J., Murakami, K. and Nakayama, K. (1991) Arg-X-Lys/Arg-Arg motif as a signal for precursor cleavage catalyzed by furin within the constitutive secretory pathway. *Journal of Biological Chemistry* 266, 12,127–12,130.

Hsu, V.P., Hossain, M.J., Parashar, U.D., Ali, M.M., Ksiazek, T.G., Kuzmin, I., Niezgoda, M., Rupprecht, C., Bresee, J. and Breiman, R.F. (2004) Nipah virus encephalitis reemergence, Bangladesh. *Emerging Infectious Disease* 10, 2082–2087.

Jack, P.J., Boyle, D.B., Eaton, B.T. and Wang, L.F. (2005) The complete genome sequence of J virus reveals a unique genome structure in the family Paramyxoviridae. *Journal of Virology* 79, 10,690–10,700.

Janke, B.H., Paul, P.S., Landgraf, J.G., Halbur, P.G. and Huinker, C.D. (2001) Paramyxovirus infection in pigs with interstitial pneumonia and encephalitis in the United States. *Journal of Veterinary Diagnostic Investigations* 13, 428–433.

Jorgensen, E.D., Collins, P.L. and Lomedico, P.T. (1987) Cloning and nucleotide sequence of Newcastle disease virus hemagglutinin-neuraminidase mRNA: identification of a putative sialic acid binding site. *Virology* 156, 12–24.

Jun, M.H., Karabatsos, N. and Johnson, R.H. (1977) A new mouse paramyxovirus. *Australian Journal of Experimental Biology and Medical Sciences* 55, 645–647.

Karabatsos, N. (ed.) (1985) *International Catalogue of Arboviruses*. American Society of Tropical Medicine and Hygiene, San Antonio, Texas.

Karron, R.A. and Collins, P.L. (2007) Parainfluenza viruses. In: Knipe, D.M., Howley, P.M., Griffin, D.E., Lamb, R.A., Martin, M.A., Roizman, B. and Straus, S.E. (eds) *Fields Virology*. Lippincott Williams & Wilkins, Philadelphia, Pennsylvania, pp. 1497–1526.

Kolakofsky, D., Pelet, T., Garcin, D., Hausmann, S., Curran, J. and Roux, L. (1998) Paramyxovirus RNA synthesis and the requirement for hexamer genome length: the rule of six revisited. *Journal of Virology* 72, 891–899.

Kolakofsky, D., Roux, L., Garcin, D. and Ruigrok, R.W. (2005) Paramyxovirus mRNA editing, the 'rule of six' and error catastrophe: a hypothesis. *Journal of General of Virology* 86, 1869–1877.

Lamb, R.A. and Parks, G.D. (2007) Paramyxoviridae: the viruses and their replication. In: Knipe, D.M., Howley, P.M., Griffin, D.E., Lamb, R.A., Martin, M.A., Roizman, B. and Straus, S.E. (eds) *Fields Virology*. Lippincott Williams & Wilkins, Philadelphia, Pennsylvania, pp. 1449–1496.

Langedijk, J.P., Daus, F.J. and van Oirschot, J.T. (1997) Sequence and structure alignment of Paramyxoviridae attachment proteins and discovery of enzymatic activity for a morbillivirus hemagglutinin. *Journal of Virology* 71, 6155–6167.

Lawrence, M.C., Borg, N.A., Streltsov, V.A., Pilling, P.A., Epa, V.C., Varghese, J.N., McKimm-Breschkin, J.L. and Colman, P.M. (2004) Structure of the haemagglutinin-neuraminidase from human parainfluenza virus type III. *Journal of Molecular Biology* 335, 1343–1357.

Li, Z., Yu, M., Zhang, H., Wang, H.Y. and Wang, L.F. (2005) Improved rapid amplification of cDNA ends (RACE) for mapping both the 5′ and 3′ terminal sequences of paramyxovirus genomes. *Journal of Virological Methods* 130, 154–156.

Li, Z., Yu, M., Zhang, H., Magoffin, D.E., Jack, P.J., Hyatt, A., Wang, H.Y. and Wang, L.F. (2006) Beilong virus, a novel paramyxovirus with the largest genome of non-segmented negative-stranded RNA viruses. *Virology* 346, 219–228.

Linne, T., Berg, M., Bergvall, A.C., Hjertner, B. and Moreno-Lopez, J. (1992) The molecular biology of the porcine paramyxovirus LPMV. *Veterinary Microbiology* 33, 263–273.

Malur, A.G., Gupta, N.K., De Bishnu, P. and Banerjee, A.K. (2002) Analysis of the mutations in the active site of the RNA-dependent RNA polymerase of human parainfluenza virus type 3 (HPIV3). *Gene Expression* 10, 93–100.

Moreno-Lopez, J., Correa-Giron, P., Martinez, A. and Ericsson, A. (1986) Characterization of a paramyxovirus isolated from the brain of a piglet in Mexico. *Archives of Virology* 91, 221–231.

Murray, K., Selleck, P., Hooper, P., Hyatt, A., Gould, A., Gleeson, L., Westbury, H., Hiley, L., Selvey, L., Rodwell, B. and Ketterer, P. (1995) A morbillivirus that caused fatal disease in horses and humans. *Science* 268, 94–97.

Myers, T.M., Pieters, A. and Moyer, S.A. (1997) A highly conserved region of the Sendai virus nucleocapsid protein contributes to the NP–NP binding domain. *Virology* 229, 322–335.

Nollens, H.H., Wellehan, J.F., Saliki, J.T., Caseltine, S.L., Jensen, E.D., Van Bonn, W. and Venn-Watson, S. (2008) Characterization of a parainfluenza virus isolated from a bottlenose dolphin (*Tursiops truncatus*). *Veterinary Microbiology* 128, 231–242.

Nylund, S., Karlsen, M. and Nylund, A. (2008) The complete genome sequence of the Atlantic salmon paramyxovirus (ASPV). *Virology* 373, 137–148.

Ortmann, D., Ohuchi, M., Angliker, H., Shaw, E., Garten, W. and Klenk, H.D. (1994) Proteolytic cleavage of wild type and mutants of the F protein of human parainfluenza virus type 3 by two subtilisin-like endoproteases, furin and Kex2. *Journal of Virology* 68, 2772–2776.

Patterson, J.B., Thomas, D., Lewicki, H., Billeter, M.A. and Oldstone, M.B. (2000) V and C proteins of measles virus function as virulence factors in vivo. *Virology* 267, 80–89.

Peeples, M.E., Wang, C., Gupta, K.C. and Coleman, N. (1992) Nuclear entry and nucleolar localization of the Newcastle disease virus (NDV) matrix protein occur early in infection and do not require other NDV proteins. *Journal of Virology* 66, 3263–3269.

Pennathur, S., Haller, A.A., MacPhail, M., Rizzi, T., Kaderi, S., Fernandes, F., Bicha, L., Schickli, J.H., Tang, R.S., Chen, W., Nguyen, N., Mathie, S., Mehta, H. and Coelingh, K.L. (2003) Evaluation of attenuation, immunogenicity

and efficacy of a bovine parainfluenza virus type 3 (PIV-3) vaccine and a recombinant chimeric bovine/human PIV-3 vaccine vector in rhesus monkeys. *Journal of General Virology* 84, 3253–3261.

Philbey, A.W., Kirkland, P.D., Ross, A.D., Davis, R.J., Gleeson, A.B., Love, R.J., Daniels, P.W., Gould, A.R. and Hyatt, A.D. (1998) An apparently new virus (family Paramyxoviridae) infectious for pigs, humans, and fruit bats. *Emerging Infectious Diseases* 4, 269–271.

Poch, O., Blumberg, B.M., Bougueleret, L. and Tordo, N. (1990) Sequence comparison of five polymerases (L proteins) of unsegmented negative-strand RNA viruses: theoretical assignment of functional domains. *Journal of General Virology* 71, 1153–1162.

Poole, E., He, B., Lamb, R.A., Randall, R.E. and Goodbourn, S. (2002) The V proteins of simian virus 5 and other paramyxoviruses inhibit induction of interferon-beta. *Virology* 303, 33–46.

Qiao, D., Janke, B.H. and Elankumaran, S. (2009) Molecular characterization of glycoprotein genes and phylogenetic analysis of two swine paramyxoviruses isolated from United States. *Virus Genes* 39, 53–65.

Qiao, D., Janke, B.H. and Elankumaran, S. (2010) Complete genome sequence and pathogenicity of two swine parainfluenzavirus 3 isolates from pigs in the United States. *Journal of Virology* 84, 686–694.

Sakai, Y., Suzu, S., Shioda, T. and Shibuta, H. (1987) Nucleotide sequence of the bovine parainfluenza 3 virus genome: its 3′ end and the genes of NP, P, C and M proteins. *Nucleic Acids Research* 15, 2927–2944.

Sato, M. and Wright, P.F. (2008) Current status of vaccines for parainfluenza virus infections. *Pediatric Infectious Diseases Journal* 27, S123–125.

Shibuta, H., Kanda, T., Hazama, A., Adachi, A. and Matumoto, M. (1981) Parainfluenza 3 virus: plaque-type variants lacking neuraminidase activity. *Infection and Immunity* 34, 262–267.

Sidhu, M.S., Menonna, J.P., Cook, S.D., Dowling, P.C. and Udem, S.A. (1993) Canine distemper virus L gene: sequence and comparison with related viruses. *Virology* 193, 50–65.

Spriggs, M.K., Johnson, P.R. and Collins, P.L. (1987) Sequence analysis of the matrix protein gene of human parainfluenza virus type 3: extensive sequence homology among paramyxoviruses. *Journal of General Virology* 68, 1491–1497.

Suzu, S., Sakai, Y., Shioda, T. and Shibuta, H. (1987) Nucleotide sequence of the bovine parainfluenza 3 virus genome: the genes of the F and HN glycoproteins. *Nucleic Acids Research* 15, 2945–2958.

Svenda, M., Berg, M., Moreno-Lopez, J. and Linne, T. (1997) Analysis of the large (L) protein gene of the porcine rubulavirus LPMV: identification of possible functional domains. *Virus Research* 48, 57–70.

Tehteh, E. and Goyal, S.M. (1988) Antibodies to parainfluenza virus type 3 in Minnesota swine. *British Veterinary Journal* 144, 613–615.

Thomas, L.H. and Stott, E.J. (1975) Comparison of three methods for sampling the bovine upper respiratory tract for viruses. *Research in Veterinary Science* 18, 227–229.

Tong, S., Li, M., Vincent, A., Compans, R.W., Fritsch, E., Beier, R., Klenk, C., Ohuchi, M. and Klenk, H.D. (2002) Regulation of fusion activity by the cytoplasmic domain of a paramyxovirus F protein. *Virology* 301, 322–333.

van Wyke Coelingh, K.L., Winter, C.C., Tierney, E.L., London, W.T. and Murphy, B.R. (1988) Attenuation of bovine parainfluenza virus type 3 in nonhuman primates and its ability to confer immunity to human parainfluenza virus type 3 challenge. *Journal of Infectious Diseases* 157, 655–662.

Wang, L., Harcourt, B.H., Yu, M., Tamin, A., Rota, P.A., Bellini, W.J. and Eaton, B.T. (2001) Molecular biology of Hendra and Nipah viruses. *Microbes and Infection* 3, 279–287.

Whelan, S.P. (2008) Response to 'Non-segmented negative-strand RNA virus RNA synthesis in vivo'. *Virology* 371, 234–237.

Yang, H.T., Jiang, Q., Zhou, X., Bai, M.Q., Si, H.L., Wang, X.J., Lu, Y., Zhao, H., He, H.B. and He, C.Q. (2011) Identification of a natural human serotype 3 parainfluenza virus. *Virology Journal* 8, 58.

Yener, G.G., Kaya, G.C., Ozturk, V. and Akdal, G. (2005) Improvement in Tc-99m HMPAO brain SPECT findings during donepezil therapy in a patient with pure akinesia. *Annals in Nuclear Medicine* 19, 607–609.

Yin, H.S., Paterson, R.G., Wen, X., Lamb, R.A. and Jardetzky, T.S. (2005) Structure of the uncleaved ectodomain of the paramyxovirus (hPIV3) fusion protein. *Proceedings of the National Academy of Sciences USA* 102, 9288–9293.

Zhao, X., Singh, M., Malashkevich, V.N. and Kim, P.S. (2000) Structural characterization of the human respiratory syncytial virus fusion protein core. *Proceedings of the National Academy of Sciences USA* 97, 14,172–14,177.

Zhu, Y.M., Shi, H.F., Gao, Y.R., Xin, J.Q., Liu, N.H., Xiang, W.H., Ren, X.G., Feng, J.K., Zhao, L.P. and Xue, F. (2011) Isolation and genetic characterization of bovine parainfluenza virus type 3 from cattle in China. *Veterinary Microbiology* 149, 446–451.

9 Porcine Rubulavirus (PoRV-LPMV)

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9.1 Historical Aspects and General Background

In the early 1980s, a seemingly new disease of pigs appeared in the state of Michoacán, Mexico. This new disease was characterized by respiratory signs, encephalomyelitis and corneal opacity in young pigs (Stephano *et al.*, 1988). It was often fatal in the early days in very young pigs, while older pigs were considerably more resistant. In older pigs, reproductive disorders were typically associated with the disease. A few years after the initial outbreak of the disease, Moreno-López *et al.* (1986) isolated a paramyxovirus. Later on, in a series of molecular studies, it was shown that the causative agent was a virus (Fig. 9.1), now classified into the rubulavirus genus, in the paramyxoviridae family. Initially, as the classification system was different at that time, this new virus was found to be a paramyxovirus, closely related to mumps virus and simian virus 5 (Berg *et al.*, 1991, 1992, 1997; Sundqvist *et al.*, 1992; Svenda *et al.*, 1997). The virus has been called La Piedad Michoacán virus (LPMV). The current recognized name is porcine rubulavirus (PoRV), sometimes with the name of the isolate such as LPMV at the end (PoRV-LPMV), as a reminder of the initial name of the virus La Piedad Michoacán virus or the town it was isolated from. In the literature, different isolates have different names, which may be confusing. But there are mainly two reference strains used in most studies, the abovementioned LPMV from 1984 and PAC-3 from 1992. The name PAC is from the Spanish abbreviation of Department of Porcine Animal Production (Producción Animal Cerdos). In addition, some isolates are called C-I to C-IV (it is unclear what this abbreviation stands for).

As has been described elsewhere, and will be described more in a later section, the typical clinical signs largely depend on the age of the pigs and thus vary a lot. Later experimental studies confirmed field observations (Allan *et al.*, 1996) showing a severe pattern in young pigs. There are some indications that the pathogenicity has changed over the years and PAC-3, for example, does not show the same severe neurological signs as LPMV from 1984 did in young pigs (Ramírez-Mendoza *et al.*, 1997; Stephano, 2002; Sánchez-Betancourt *et al.*, 2008). The evolution of PoRV has provided a number of subgroups, which are suggested to be three based on the HN gene (Sánchez-Betancourt *et al.*, 2008). For the other genes the sequence information is scarce and therefore any general conclusion on viral evolution over time cannot be made. However, observation of the phylogenetic

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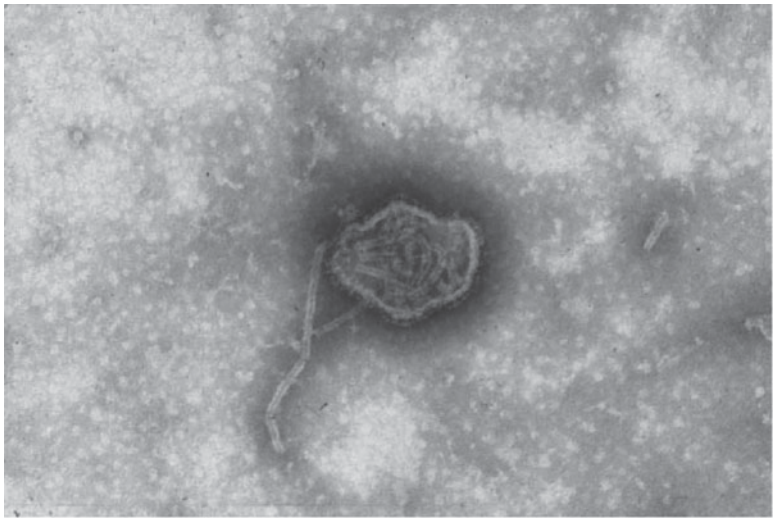

Fig. 9.1. Electron microscope picture of PoRV-LPMV (from Moreno-López *et al.,* 1986), typically showing a damaged virion with the herring-bone nucleocapsid leaking out.

tree in the paper by Sánchez-Betancourt (2008) suggests that there may be more subtypes/genotypes. For example, LPMV and PAC4 make up one, PAC6-9 one, PAC2-3 one, CI-III one and finally C-IV another one.

9.2 Origin and Maintenance of the PoRV

The origin of PoRV has been discussed since its first appearance in pigs. Fruit bats are known reservoirs of viruses, including several recently appearing paramyxoviruses such as Nipah virus, Hendra virus, Menangle virus, Tioman virus and Mapuera virus, so fruit-eating bats have been suspected to be the host of PoRV. In fact, Mapuera virus is rather closely related to PoRV both on a sequence level and in genomic organization (Wang *et al.*, 2007). This virus was isolated from a fruit bat in Brazil in 1979, when PoRV first appeared. They are however quite divergent in sequence and too far away geographically to be a direct transmission. But it clearly shows that related viruses were in circulation in the Americas at the time. Strong evidence for fruit-eating bats being the reservoirs of PoRV was provided by Salas-Rojas *et al.* (2004). They showed that one sample from several collected fruit bats from different species in Mexico was sero-positive for PoRV. Even if it was only one out of many, this indicated fruit bats in the region can carry PoRV. It is not clear if this finding is important for the epidemiology of the virus, but it indicates that the virus originally came from fruit bats, even if other reservoirs cannot be ruled out. It is also possible that the virus has been present in pigs in Mexico for a long time, but changed its pathogenicity in the early 1980s.

The most likely reservoir of the virus at this day and from the original introduction is likely persistently infected pigs or pigs with milder clinical symptoms. Although older pigs are not very susceptible in terms of disease signs, this does not mean that they are resistant to infection. On the contrary, several studies suggest that older pigs are susceptible to infection and can therefore maintain the virus in the pig population. Furthermore, serological surveillance studies indicate that the virus is widespread in the pig population (Escobar-López *et al.*, 2011). Taken together, the virus is circulating freely in the pig population and a wild reservoir has minor importance for the maintenance of PoRV in Mexico.

9.3 Current Situation

The situation in Mexico is still problematic and several possible serotypes are co-circulating. A recent study by Escobar-López *et al.* (2011) showed that the overall sero-prevalence is quite high and differs between the states. For example, in the state of Guanajuato, the sero-prevalence has been as high as 29.7% (Morilla *et al.*, 2002), which was confirmed by Escobar-López *et al.* (2011). There is also a typical pattern where different isolates predominate in different states, while some co-circulate in others (Escobar-López *et al.*, 2011). Some studies suggest that there are at present subgroups that share serological and genetic similarities. The disease is endemic in Mexico, but has not been found or described elsewhere. In Mexico, the presence of the virus/disease is well correlated with the density of pig production, since states with large production also have more problems with the disease (Escobar-López *et al.*, 2011). In fact, PoRV is considered to be one of the most severe diseases affecting the pig industry.

9.4 Clinical Picture and Pathogenesis

As noted before, the clinical signs are variable and depend on the age of the pig. Piglets 2–15 days old are most susceptible, and the clinical signs are quite sudden in onset. The main characteristics of the disease are encephalomyelitis, pneumonia and corneal opacity. The disease involves infection of the brain and other parts of the central nervous system, producing nervous signs: ataxia, weakness, rigidity in the hind legs, muscle tremor, involuntary movements etc. After the appearance of the first signs, piglets usually die within 48 hours (Stephano *et al.*, 1988). It has been suggested that the virus is responsible for an increased number of stillbirths and mummified foetuses (Stephano and Gay, 1984). Some boars develop orchitis, epididymitis and later on unilateral or bilateral testicle atrophy. The semen quantity and quality is affected. Abscess on the epididymis head histologically corresponding to spermatic granulomas was also observed. The pathobiology and clinical signs of the infection were reviewed by Stephano *et al.* (1988; Stephano, 1999).

The main disease signs are also reproducible experimentally in piglets, pregnant gilts and boars. The outcome of experimental infection in animals at different age is described below:

9.4.1 Experimental infection in piglets

The clinical outcome of experimental infection in piglets at the age of 3 or 17 days has been described by Hernández-Jáuregui *et al.* (2004). The piglets killed sequentially at different days post-infection showed clinical pathological signs of the central nerve system (CNS) (ataxia, muscle tremor), increased respiratory rate, some of them developed corneal opacity, and several pigs died. The clinical sigs in the older pigs were less severe than in the younger animal. Sequential post mortem examination revealed gross and microscopic lung and brain lesions. Neural lesions commenced in the olfactory pathway, and subsequently occurred in other fore mid- and hind-brain regions. Brain lesions were associated with PoRV antigens in neurons and macrophages; viral antigens were demonstrated in pneumocytes and intra-septal and intra-alveolar macrophages in pigs that had gross lung lesions.

These results indicate that 3-day-old pigs are more susceptible to PoRV infection than 17-day-old pigs and that, following intranasal and intra-conjuntival inoculation, PoRV can gain anterograde access to the brain via the olfactory pathway. This finding may explain the rapid onset of nervous signs in young pigs during spontaneous outbreaks of PoRV infection. The histopathological lesions and viral distribution in different tissues in most of the experimental infected piglets were in agreement with observations on age-dependent susceptibility in naturally infected piglets (Stephano *et al.*, 1988; Allan *et al.*, 1996; Kennedy *et al.*, 2002). In experimentally

infected animals both viral proteins and viral nucleic acid could be detected. Using a polymerase chain reaction (PCR) technique the viral P gene was detected in different organs including the brain (Berg *et al.*, 1992).

9.4.2 Experimental infection in pregnant gilts

The gilts at 6 or 10 weeks of gestation were experimentally infected and killed at 8 or 15 weeks of gestation or after natural parturition (Hernández-Jáuregui *et al.*, 2004). Oro-nasal exposure of pregnant gilts to PoRV resulted in virus-induced reproductive failure. The gilts show focal congestion and haemorrhages in the placenta and endometrium. Abnormal pregnancies were found on gilts and PoRV was isolated from lungs, tonsils, ovaries, placenta, uterus and lymph nodes. The allantoic fluid from one gilt, at 9.5 weeks of gestation and 3.5 weeks after inoculation, was positive for virus isolation. Many foetuses were smaller than normal and had dermal ecchymoses. Dead foetuses, brown in colour and with autolysis, were frequently found and were dehydrated or mummified. Early signs of foetal damage were diffuse haemorrhages on the skin and diminished size, compared with no affected foetuses in the same or parallel gravid horn. Older foetuses that died in uteri were also found to be dehydrated and with advance autolysis (mummies). Virus isolation from brain and lung tissues was possible from several foetuses. These observations supported previous clinical studies indicating susceptibility of pregnant swine after natural outbreaks of PoRV.

The anatomy of the porcine placenta would seem to preclude direct transfer of virus between maternal and foetal circulation. In the absence of immunity, gilts and sows may be susceptible to viral infection, resulting in stillbirths, mummies and abortions. It appears that the placenta and endometrium are not selective sites of virus replication and cell damage. These observations supported previous clinical studies indicating susceptibility of pregnant swine after natural outbreaks of PoRV. The results indicated that after experimental infection, PoRV can replicate in tissues of seronegative pregnant gilts, cross the placenta, and cause foetal death and mummification. In experimentally infected animals both viral proteins and viral nucleic acid could be detected. Using a polymerase chain reaction (PCR) technique the viral P gene was detected in different organs, including the brain (Berg *et al.*, 1992).

9.4.3 Experimental infection in boars

Boars infected with PoRV at 9 months of age were killed sequentially on different days post inoculation (Ramírez-Mendoza *et al.*, 1997; Moreno-Lopez and Hernández-Jáuregui 2002; Hernández-Jáuregui, 2003). In general, post-mortem examinations of the reproductive tract showed swelling, severe fibrosis and nodules in the head of the epididymis and some testes were atrophic with degeneration of seminiferous tubules and interstitial mononuclear cell infiltration. Histopathological alterations included formation of spermatic granulomas and vacuolar degeneration of ductular epithelium. In immunofluorescence and immunohistochemistry assays using a monoclonal antibody against the NP protein, PoRV antigens were also found in the head of the epididymis. The results indicate that porcine rubulavirus can cause severe epididymitis, orchitis and reduced semen quality in sexually mature boars. The porcine rubulavirus is closely related to human mumps virus, sharing 41–47% protein sequences homologies (Sundqvist *et al.*, 1990, 1992; Berg *et al.*, 1991, 1992). Mumps virus infection in man causes epididymitis, orchitis, parotitis, pancreatitis and meningoencephalitis (Gnann, 1992), and similar organ tropism (brain, testicles, epididymis) is a characteristic feature of PoRV infection. Based on these results and the close genetic relationship to mumps virus in humans, it is of particular interest to correlate the pathological lesions observed in PoRV-infected boars with those of mumps virus infection in humans. In both cases the

infection is characterized by epididymitis, orchitis and meningoencephalitis (Gnann, 1992; Ramírez-Mendoza *et al.*, 1997). In mumps virus and PoRV infections, the epididymitis may be of a short duration, followed by a relatively rapid restoration of affected tissues. In view of the genomic relatedness of porcine rubulavirus to mumps virus, it is not surprising that both viruses have tropisms for a similar range of tissues. In parotitis, epididymo-orchitis occurs in approximately 25–30% of post-pubertal men with mumps infection and pancreatitis is also present (Gnann, 1992).

Based on these results, it is concluded that the lesions in the epididymis and testicles of PoRV-infected boars may resemble those of mumps virus infection in humans. Considering the similarities of the PoRV lesions in the epididymis and the lack of more relevant information of the lesions in the human with mumps infection, PoRV infection in sexually mature boars may therefore be a useful model for mumps virus infection in the reproductive tract of post-pubertal human males.

9.5 Molecular Biology

The observation that PoRV appeared to have changed its pathogenicity is intriguing. The viral genetic background of its molecular mechanism is still unclear but would be interesting to study in more detail. The initial pathogenicity was considered to be very high in young pigs with very unpleasant disease signs. The HN protein may be a key factor of pathogenicity, as suggested by Sánchez-Betancourt *et al.* (2008). They performed a thorough analysis of selected viruses with different pathogenicity from low neurovirulence to more severe form of the diseases, and concluded that this difference is associated with HN gene sequences. They observed that the PAC 6–9 group, being closely related genetically, also were more neurovirulent. This led to the conclusion that the HN gene might be responsible for the increased neurovirulence (Sánchez-Betancourt *et al.*, 2008). But, as they pointed out, there are likely to be more factors of relevance for the differences in pathogenicity. It might also be a multigenic property, such that the HN gene and some immune evasion mechanisms, mediated from other viral genes, cooperate to give PoRV its highly pathogenic properties.

There are very few other known attempts to figure out the viral genetic background behind the pathogenicity determinants for PoRV. But the virus has been characterized in some detail molecularly and in cell culture system. A number of experimental infection studies have been performed, but no comparative studies in the sense that a known genetic factor can be linked to the high neurovirulence of PoRV have been conducted. The whole genome of the virus has been determined for one isolate from 1984 LPMV (Berg *et al.*, 1991, 1992, 1997; Sundqvist *et al.*, 1992; Svenda *et al.*, 1997; Wang *et al.*, 2007). In addition, the HN (Sánchez-Betancourt *et al.*, 2008) and F genes (Berg *et al.*, 1997) have been sequenced from a number of isolates. But in total, we know very little on the genetic variation over time of this virus, and further studies would be valuable. The genome follows the standard organization of viruses in the mononegavirales, assuming that the functions of the genes are similar to other related viruses. The full-length genome sequence was determined in 2007, including the gene-ends, and was assembled with the sequence data from earlier reported genes (Wang *et al.*, 2007). The order of the genes from the 3′-end is NP-P/V/C-M-F-HN-L (Fig. 9.2). However, one peculiar notification that LPMV shares with the related Mapuera virus of fruit bats was that the P-gene has an open reading frame of a putative C-protein (Berg *et al.*, 1992; Wang *et al.*, 2007). This protein has an internal AUG start codon in a different reading frame from P/V. Similar between these viruses is also that the P-mRNA needs to be edited to 'open-up' the C-terminal part of the P protein at its editing site (Berg *et al.*, 1992). The unedited mRNA expresses the V protein. A third product, sometimes called I, may also be expressed via insertion of one G at the editing site. This putative protein has an

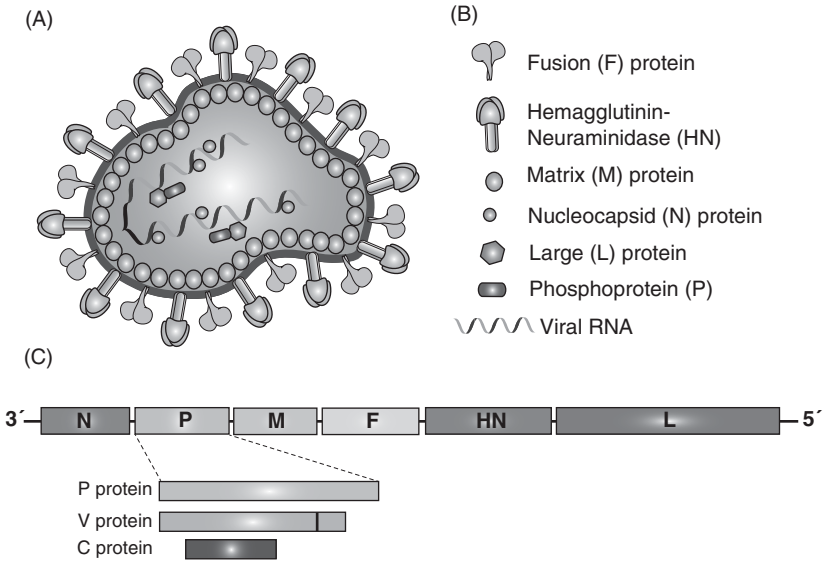

Fig. 9.2. The genomic organization and proteins of PoRV-LPMV. The full genome of PoRV-LPMV is 15,180 nucleotides long and has the protein-coding genes NP-P/V/C-M-HN-F-L.

early stop codon and has only six amino acids from the editing site (Berg *et al.*, 1992). No studies have been conducted to address the functional relevance of the editing and differential expression of the P, V and I proteins. Unpublished data indicated that during persistent infection in cell culture there was significantly more expression of V than P protein. The data have however not been verified in persistently infected animals or in any other *in vivo* situation. If this is true during infection, this may well be related to the known function of the V protein of related viruses of inhibiting type I interferon (Versteeg and García-Sastre, 2010). It is not know whether the V protein of PoRV has the ability to block the IFN system, but ongoing work in several laboratories may soon answer this question.

What has been studied, however, is how these proteins interact with other viral proteins such as NP and L (Svenda *et al.*, 2002). It was shown that both V and P interacted strongly with NP, but surprisingly not with their common part but instead with their unique C-terminal parts. It can thus be speculated that they compete with the binding of NP and that this has some function during the infection cycle, or more interesting, with some function of the possible interferon down-regulation. But it was also shown that the region of NP that V and P interacted with was different. This may indicate that they interact with different forms of NP, or in fact both interact simultaneously with NP. It was also shown that they interact with the polymerase protein and are thus likely parts of different polymerase complexes. It can also be noted that it appears that they interact with some cellular proteins (Fig. 9.2; Svenda *et al.*, 2002). These have not been identified as yet, but they may be of interest for further studies.

As mentioned earlier, considerable effort has been put into the analysis of the HN gene. Reyes Leyva *et al.* (1997) demonstrated the substrate specificity of the virus was of so-called NeuAc2.3-Gal type, and that this was correlated to the cell specificity of the virus. Later more detailed studies on pathogenic variants of the virus suggested that a series of mutations in the HN gene could be responsible, or at least part of the changed pathogenicity between isolates (Sánchez-Betancourt *et al.*, 2008). In particular, residues that are supposedly important

for receptor accessibility may increase the neurovirulence. In support for this suggestion, studies on a related virus, mumps virus, are mentioned (Reyes-Leyva *et al.*, 2007).

9.5.1 The F-protein

As far as we are aware, the sequences of only two F-genes have been determined. The first one from the above mentioned LPMV and the other called San Fandilla from 1988 (Berg *et al.*, 1997). They were very similar, having only 16 differences at the nucleotide level, giving rise to four amino acid differences. In many other paramyxoviruses, this protein needs to be cleaved to be functional, being a functional homologue of the more famous haemagglutinin (HA) of the influenza virus. The cleavage site for the F-genes of PoRV is HRKKR-F. These polybasic amino acid cleavage sites are similar to avian paramyxoviruses, such as Newcastle disease virus, and are considered to be a hallmark of highly pathogenic viruses in comparison to low pathogenic viruses that have only one R at the cleavage site. In mammalian viruses that carry this functional homologue (F or HA) that correlation is not as clear-cut. Nevertheless, it would be interesting to compare, for example, the F-genes of these highly neurovirulent isolates to the less neurovirulent ones such as PAC-3.

9.6 Diagnostic Tools

At present, to our knowledge, only serological tests and classical virus isolation techniques are available for diagnostic purposes (McNeilly *et al.*, 1997; Nordengrahn *et al.*, 1999; Gonzáles Vega *et al.*, 2002). In some publications a standard haemagglutination-inhibition assay has commonly been used (Escobar-López *et al.*, 2011). To study the virus in detail and to improve the diagnosis and immune response to a vaccine, an ELISA was developed using a set of monoclonal antibodies. The panel of monoclonal antibodies was against the LPMV nucleoprotein (NP) and haemagglutinin-neuraminidase (HN) proteins, respectively. Three main epitopes on the NP were found and all the NP monoclonal antibodies were able to immunoprecipitate the NP from infected cells and disrupted virions. The monoclonal antibody (Mab) against the HN protein could prevent infection and haemagglutination, and is thus directed towards a 'neutralizing' epitope (Sundqvist *et al.*, 1990). Later on, a blocking B-ELISA against LPMV was developed to facilitate screening of large numbers of swine sera (Nordengrahn *et al.*, 1999). The assay is based on a Mab against HN glycoprotein, which was shown to be one of two proteins important for protective immunity against LPMV.

In several studies (McNeilly *et al.*, 1997; Ramirez-Mendoza *et al.*, 1997; Gonzáles Vega *et al.*, 2002), and compared with other conventional serological tests, this B-ELISA was shown to be robust, highly specific and sensitive, and can detect infection at an early stage in pigs. It is therefore suitable for screening a large number of sera. It was recommended that a B-ELISA system should be used in epidemiological studies in Mexico and should also be considered for use in outbreaks of undiagnosed diseases in pigs in other Central and South American countries and in the USA. This assay will be practical for monitoring the results of vaccination programmes, which in the near future will be routine in intensive pig-production units in Mexico.

In addition, in publications aimed at answering virus biological questions, classical PCR technology, sometimes including a nested PCR step, has been described and used (Berg *et al.*, 1992, Wiman *et al.*, 1998; Cuevas *et al.*, 2009). But this has not been routinely used for diagnostic purposes. A real-time PCR method is not yet available, but development work is in progress. This technique has many advantages over classical PCR, such as better sensitivity and specificity (Belák and Thorén, 2001), and gives the possibility to quantify the exact levels of viral RNA. Another advantage is that the procedure can be combined with other possible agents to give a thorough diagnostic tool. In addition, the procedure is much less

prone to contamination problems. Ideally, the real-time method in development will be implemented and used routinely for diagnosis of PoRV-LPMV. The system is based on the P-gene and is a standard Tacman system (Cuevas *et al.*, in progress). The sensitivity is high (about ten copies of viral RNA) and as is the specificity. It works on all tested isolates that have been established, which includes the very early isolates from the early 1980s up to some very recent isolates. But since we know very little about the genetic variation between isolates, we do not know at present if the assay works on all isolates that are present, and how well it will work in the future. Likely the assay, as all other primer-based assays, will need some updating in the future.

9.7 Future Aspects

Considering the importance of the disease caused by PoRV, this virus deserves more research attention. But, because it appears only in Mexico research funds are scarce and therefore research has been rather limited. Obviously a good protective vaccine would be helpful in the effort to control this disease. Ideally it would be based on the HN and F proteins using a subunit vaccine such as the 'ISCOM' concept (Morein *et al.*, 1984). As far as we know, there have been some efforts to develop such a vaccine. Fruit-eating bats have long been suspected of carrying different viruses and of being a major player in its epidemiology. As mentioned above, antibodies to PoRV-LPMV were found in one bat, indicating that this may very well be true (Salas-Rojas *et al.*, 2004). It would be very interesting to further pursue this line of research and, for example, use the newly developed real-time RT-PCR system to investigate more animals. As mentioned earlier, we have limited sequence information about more isolates of this virus. Several isolates of PoRV need to be fully characterized to gain insights into the genetic nature of circulating viruses.

References

Allan, G.M., McNeilly, F., Walker, I., Linne, T., Moreno-Lopez, J., Hernández, P., Kennedy, S., Carroll, B.P., Herron, B., Foster, J.C. and Adair, B. (1996) A sequential study of experimental porcine paramyxovirus (LPMV) infection of pigs: immunostaining of cryostat sections and virus isolation. *Journal of Veterinary Diagnostic and Investigation* 8, 405–413.

Belák, S. and Thorén, P. (2001) Molecular diagnosis of animal diseases: some experiences over the past decade. *Expert Review of Molecular Diagnostics* 1, 434–443.

Berg, M., Sundqvist, A., Moreno-Lopez, J. and Linne, T. (1991) Identification of the porcine paramyxovirus LPMV matrix protein gene: comparative sequence analysis with other paramyxoviruses. *Journal of General Virology* 72, 1045–1050.

Berg, M., Hjertner, B., Moreno-Lopez, J. and Linne, T. (1992) The P gene of the porcine paramyxovirus LPMV encodes three possible polypeptides P, V and C: the P protein mRNA is edited. *Journal of General Virology* 73, 1195–1200.

Berg, M., Bergvall, A.C., Svenda, M., Sundqvist, A., Moreno-Lopez, J. and Linne, T. (1997) Analysis of the fusion protein gene of the porcine rubulavirus LPMV: comparative analysis of paramyxovirus F proteins. *Virus Genes* 14, 55–61.

Cuevas, J.S., Rodriguez-Ropón, A., Kennedy, S., Moreno-Lopez, J., Berg, M. and Hernández-Jáuregui, P. (2009) Investigation of T-cell responses and viral mRNA persistence in lymph nodes of pigs infected with porcine rubulavirus. *Veterinary Immunology and Immunopathology* 127, 148–152.

Escobar-López. A.C., Rivera-Benitez, J.F., Castillo-Juárez, H., Ramírez-Mendoza, H., Trujillo-Ortega, M.E. and Sánchez-Betancourt, J.I. (2011) Identification of nitgenic variants of the porcine rubulavirus in sera of field swine and their seroprevalence. *Transboundary and Emerging Diseases* 59, 416–420.

Gnann, J.W. (1992) Mumps. In: Wyngaarden, J.B., Smith, L.H. Jr and Benett, J.C. (eds) *Cecil Texbook of Medicine*, 19th edn. WB Saunders, Philadelphia, Pennsylvania, p. 1829.

Gonzáles Vega, D., Diosdado, F., Nordengrann, A., Mercado, M., Hernández-Jáuregui, P., Moreno-Lopez, J. and Morilla, A. (2002) In: Morilla, A., Yoon, K.-J. and Zimmerman, J.J. (eds) *Trends in Emerging Viral Diseases of Swine*. Iowa State University Press, Ames, Iowa, pp. 55–57.

Hernández-Jáuregui and Alvarez, P. (2003) *The Pathogenesis of the Porcine Rubulavirus (LPMV)*. Acta Universitatis Agriculturae Sueciae. Veterinaria 155. Swedish University of Agricultural Sciences, Uppsala, Sweden.

Hernández-Jáuregui, P., Ramirez-Mendoza, H., Mercado, G.C., Moreno-Lopez, J. and Kennedy, S. (2004) Experimental porcine rubulavirus (La Piedad-Michoacan virus) infection in pregnant gilts. *Journal of Comparative Pathology* 130, 1–6.

Kennedy, S., Herron, B., Hernández-Jáuregui, P., Allan, G., Kirk, J. and Moreno-Lopez, J. (2002) Neuropathology of porcine rubulavirus infection. In: Morilla, A., Yoon, K.-J. and Zimmerman, J.J. (eds) *Trends in Emerging Viral Diseases of Swine*. Iowa State University Press, Ames, Iowa, pp. 87–91.

McNeilly, F., Walker, I., Allan, G.M., Foster, J.C., Linné, T., Merza, M., Hernández, P., Kennedy, S. and Adair, B. (1997) A comparative study on the use of virus and antibody detection techniques for the diagnosis of La Piedad Michoacan paramyxovirus (LPMV) infection in pigs. *Journal of Veterinary Diagnostic and Investigation* 9, 3–9.

Morein, B., Sundquist, B., Höglund, S., Dalsgaard, K. and Osterhaus, A. (1984) ISCOM, a novel structure for antigenic presentation of membrane proteins from enveloped viruses. *Nature* 308, 457–460.

Moreno-Lopez, J. and Hernández-Jáuregui, P. (2002) Are the lesions in the epididimis of boars infected with porcine rubulavirus (LPMV) similar to those of mumps virus in humans? In: Morilla, A., Yoon, K.-J. and Zimmerman, J.J. (eds) *Trends in Emerging Viral Diseases of Swine*. Iowa State University Press, Ames, Iowa, pp. 91–96.

Moreno-Lopez, J., Correa-Girón, P., Martinéz, A. and Ericsson, A. (1986) Characterization of a paramyxovirus isolated from the brain of a piglet in Mexico. *Archives of Virology* 91, 221–231.

Morilla, A., González-Vega, D., Estrada, E. and Diosdado, F. (2002) Seroprevalence of blue eye disease. In: Morilla, A., Yoon, K.-J. and Zimmerman, J.J. (eds) *Trends in Emerging Viral Infections of Swine*. Iowa State University Press, Ames, Iowa, pp. 59–64.

Nordengrahn, A., Svenda, M., Moreno-Lopez, J., Bergvall, A., Hernández, P., McNeilly, F., Allan, G. and Merza, M. (1999) Development of a blocking ELISA for screening antibodies to porcine rubulavirus, La Piedad Michoacan Virus. *Journal of Veterinary Diagnostic and Investigation* 11, 319–323.

Ramírez-Mendoza, H., Hernández-Jáuregui, P., Reyes-Leyva, J., Zenteno, E., Moreno-López, J. and Kennedy, S. (1997) Lesions in the reproductive tract of boars experimentally infected with porcine rubulavirus. *Journal of Comparative Pathology* 117, 237–252.

Reyes-Leyva, J., Espinosa, B., Hernández, J., Zenteno, R., Vallejo, V., Hernández-Jáuregui, P. and Zenteno, E. (1997) NeuAc alpha 2,3gal-glycoconjugate expresion determines cell susceptibility to the porcine rubulavirus LPMV. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 118, 327–332.

Reyes-Leyva, J., Banos, R., Borraz-Argüello, M., Santos-López, G., Rosas, N., Alvaro, G., Herrera-Camacho, I., Vallejo, V. and Tapia-Ramirez, J. (2007) Amino acid change 335 E to K affects the sialic-acid-binding and neuraminidase activities of the Urabe AM9 mumps virus hemagglutinin-neuraminidase glycoprotein. *Microbes and Infection* 9, 234–240.

Salas-Rojas, M., Sánchez-Hernández, C., Romero-Almaraz, M.L., Schnell, G.D., Schmid, R.K. and Aguilar-Setién, A. (2004) Prevalence of rabies and LPM paramyxovirus antibody in non-hematophagous bats captured in the Central Pacific coast of Mexico. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 98, 577–584.

Sánchez-Betancourt, J.I., Santos-Lopez, G., Alonso, R., Doporti, J.M., Ramirez-Mendoza, H., Mendoza, S., Hernández, J., Reyes-Leyva, J. and Trujillo, M.E. (2008) Molecular characterization of the hemagglutinin-neuraminidase gene of porcine rubulavirus isolates associated with neurological disorders in fattening and adult pigs. *Research in Veterinary Sciences* 85, 356–367.

Stephano, H.A. (1999) Blue eye disease. In: Straw, B.E., D'Allaire, S., Mengeling, W.L. and Taylor, D.J. (eds) *Diseases of Swine*. Iowa State University Press, Ames, Iowa, pp. 103–112.

Stephano, H.A. (2002) Blue eye disease: clinical signs and lesions. In: Morilla, A., Yoon, K.-J. and Zimmerman, J.J. (eds) *Trends in Emerging Viral Diseases of Swine*. Iowa State University Press, Ames, Iowa, pp. 47–50.

Stephano, H.A. and Gay, M. (1984) Experimental studies on a new viral disease in pigs called 'blue eye', characterized by encephalitis and corneal opacity. *Proceedings of the 8th International Pig Veterinary Society Congress*, Ghent, Belgium, p. 71.

Stephano, H.A., Gay, G.M. and Ramirez, T.Z. (1988) Encephalomyelitis, reproductive failure and corneal opacity (blue eyes) in pigs, associated with a paramyxovirus infection. *Veterinary Record* 122, 6–10.

Sundqvist, A., Berg, M., Hernández-Jáuregui, P., Linné, T. and Moreno-Lopez, J. (1990) The structural proteins of a porcine paramyxovirus (LPMV). *Journal of General Virology* 71, 609–613.

Sundqvist, A., Berg, M., Moreno-Lopez, J. and Linné, T. (1992) The haemagglutinin-neuraminidase glycoprotein of the porcine paramyxovirus LPMV: comparison with other paramyxoviruses revealed the closest relationship to simian virus 5 and mumps virus. *Archives of Virology* 122, 331–340.

Svenda, M., Berg, M., Moreno-Lopez, J. and Linné, T. (1997) Analysis of the large (L) protein gene of the porcine rubulavirus LPMV: identification of possible functional domains. *Virus Research* 48, 57–70.

Svenda, M., Hjertner, B., Linné, T. and Berg, M. (2002) Both the P and V proteins of the porcine rubulavirus LPMV interact with the NP protein via their respective C-terminal unique parts. *Virus Research* 83, 31–41.

Versteeg, G.A. and García-Sastre, A. (2010) Viral tricks to grid-lock the type I interferon system. *Current Opinions in Microbiology* 13, 508–516.

Wang, L.F., Hansson, E., Yu, M., Chua, K.B., Mathe, N., Crameri, G., Rima, B.K., Moreno-Lopez, J. and Eaton, B.T. (2007) Full-length genome and genetic relationship of two paramyxoviruses isolated from bat and pigs in the Americas. *Archives of Virology* 152, 1259–1271.

Wiman, A.C., Hjertner, B., Linné, T., Herron, B., Allan, G., McNeilly, F., Adair, B., Moreno-Lopez, J. and Berg, M. (1998) Porcine rubulavirus LPMV RNA persists in the central nervous system of pigs after recovery from acute infection. *Journal of Neurovirology* 5, 545–552.

10 Bovine Respiratory Syncytial Virus

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10.1 Historical Perspective

In the autumn of 1955 an outbreak of acute upper respiratory illness, characterized by coughing, sneezing and mucopurulent nasal discharge, and commonly know as 'coryza', occurred in a colony of 20 otherwise normal chimpanzees at the Walter Reed Army Institute of Research in Washington, DC. In 1956, Morris and colleagues reported the recovery of a cytopathogenic agent from some of the affected animals in this outbreak, and named it 'chimpanzee coryza agent' (CCA) (Morris *et al.*, 1956). They conducted preliminary pathogenicity studies and reported the reproduction of coryza in chimps inoculated intranasally with cultures of the agent, but no disease in mice, hamsters, rabbits or guinea pigs inoculated by multiple routes. Moreover, they reported coryza-like symptoms in an in-contact laboratory worker, and complement-fixing (CF) antibodies to CCA in adolescents and young adults, providing the first evidence of human infection with CCA, or a closely related agent (Morris *et al.*, 1956). In 1957, Chanock and colleagues reported on isolations of agents indistinguishable from CCA from the throats of paediatric patients with severe lower respiratory tract disease at Johns Hopkins University Hospitals in Baltimore, Maryland (Chanock *et al.*, 1957). They observed that at least one of their isolates, the 'Long' virus, produced prominent syncytia in cell culture. In a follow-up preliminary epidemiologic study, Chanock and Fineberg (1957) confirmed and extended the seminal observations of Morris and colleagues, and reported the high prevalence of seroconversion to the human isolates in a significant portion of outpatients with respiratory infections, as well as in clinically normal individuals. In view of the pathogenicity in chimps, the shared and indistinguishable characteristics of chimp and human isolates, and the association with respiratory disease in humans, they proposed the still-used name 'respiratory syncytial virus' (RSV). Subsequently, the worldwide high prevalence and importance of human RSV (hRSV) was rapidly established, and currently hRSV is considered a (the) major cause of respiratory disease in infants, that not uncommonly requires hospitalization (Hall *et al.*, 2009). At the time of writing, despite nearly 50 years of research, that began with the development and failed application of an apparently disease-enhancing formalin-inactivated vaccine in children in the late 1960s, there still is not a single hRSV vaccine in clinical use (Power, 2008; Collins and Melero, 2011).

In 1968, in England, Doggett and colleagues provided the first evidence that an

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agent closely related to hRSV existed and caused disease in cattle. They demonstrated neutralizing antibodies against two strains of hRSV in sera of adult cattle, and of calves convalescent from respiratory disease of unknown aetiology, and suggested that a RSV-like agent could be associated with the bovine respiratory disease complex (BRDC) (Doggett *et al.*, 1968). In 1970, bovine RSVs (bRSV) were first isolated in Switzerland (Paccaud and Jacquier, 1970), Belgium (Wellemans *et al.*, 1970) and Japan, where it was first called Nomi virus, and subsequently, RSV (Inaba *et al*, 1970a, 1970b). Following shortly thereafter, bRSVs were isolated in England in 1971 (Jacobs and Edington, 1971), and for the first time in North America in 1974 (Rosenquist, 1974). Subsequently, bRSV has been documented worldwide as a major cause of calfhood pneumonia and an aetiologic agent in BRDC (Baker *et al.*, 1992). In contrast to the situation in human medicine, vaccine development for bRSV, which began in the late 1970s, has resulted in the commercial availability of a variety of intranasal and parenteral modified-live and inactivated vaccines that have proven to be efficacious in robust challenge models (Brodersen, 2010). A major focus of applied vaccine research today is the use of vaccines in young bRSV antibody-positive calves (Ellis *et al.*, 2010).

10.2 The Virion and its Proteins

Bovine respiratory syncytial virus is a representative of the *Pneumovirinae* in the family *Paramyxoviridae* (Valarcher and Taylor, 2007). bRSV is very closely related genetically and antigenically to hRSV, with percent amino acid identity ranges from 30% for the G protein to 93% for the N protein (Valarcher and Taylor, 2007). Much of the current thinking regarding the molecular virology of bRSV is extrapolated from the more studied hRSV. The pleomorphic, spherical bRSV virion consists of an envelope and nucleocapsid. As with all enveloped viruses, the lipid envelope of the bRSV virion comprises virally derived and host-cell components. Viral components include two glycosylated, G and F (fusion) proteins, and a small hydrophobic (SH) protein, the transmembrane moieties of which are associated with a matrix or M protein, which forms a layer on the inner face of the envelope. The heavily glycosylated G protein appears to exist as a trimer on the surface of the virion (Valarcher and Taylor, 2007). Historically, the G protein has been implicated in the attachment of RSVs to host target cells, based on the observation that monoclonal antibodies against the G protein blocked this effect (Levine *et al.*, 1987). The G protein is also expressed as a secreted form that is unique among respiratory viruses, and may help RSVs escape antibody responses (Bukreyev *et al.*, 2008). The G protein is variable amongst bRSV isolates. This together with the high carbohydrate content comprising 'mucin-like' domains that may shield it from antibody responses should make it a poor antigen. However, this protein has cysteine-rich conserved amino acids in an internal hydrophobic region that are thought by some to form an immunodominant epitope that has been proposed as a major target of protective immune responses (Valarcher and Taylor, 2007). The other glycosylated envelope protein, the fusion or F protein, mediates penetration of the virus into the target cell. More recent studies indicate that it is also involved in attachment through interaction with the cellular protein known as nucleolin (Tayyari *et al.*, 2011). It is also responsible for the fusion of infected cells during the process of typical syncytium formation *in vitro* and *in vivo*. In contrast to the G protein, the F protein is highly conserved among bRSV isolates and between bRSV and hRSV (Valarcher and Taylor, 2007). Given its indispensability in viral replication, neutralizing antibodies against the F protein are protective (Valarcher and Taylor, 2007). The small hydrophobic or SH protein is unique to the *Pneumovirinae*, and although it is an integral membrane protein, it is not essential for virus replication *in vivo* or *in vitro*. Its function is currently not well defined (Collins and Melero, 2011). The RSV M protein, which has little sequence

relatedness to M proteins of the other paramyxoviruses, plays a role in budding and formation of new viral particles. The host-cell components of the envelope are poorly described in bRSV, but likely to include actin and caveolin-1, as with hRSV, and perhaps MHC I class I molecules (Valarcher and Taylor, 2007).

The envelope encloses a helical nucleocapsid, or ribonucloprotein (RNP), comprising the nucleoprotein (N), a viral RNA-dependent polymerase or L protein, and a co-factor for the polymerase, the phoshoprotein or P protein, which together are tightly bound to a negative-sense genomic RNA, and its positive-sense replicative intermediate or 'antigenome' (Valarcher and Taylor, 2007). The bRSV genome is approximately 15 kb in length and has 10 genes organized as 10 open reading frames (ORFs) in the order 3′-NS1-NS2-N-P-SH-F-G-M2-L-5′. These ORFs are transcribed into 10 separate mRNAs and each is translated into a single polypeptide, except for M2 mRNA. This has an upstream ORF that is translated into the M2-1 protein and a second overlapping downstream ORF that is translated into the M2-2 protein (Collins and Melero, 2011). These latter two proteins belong to a complex of matrix proteins and are novel regulatory peptides or RNA synthesis factors, known as the transcriptional antitermination factor (M2-1) and RNA regulatory protein (M2-2). RSVs are distinct from other members of the *Paramyxoviridae* in having three matrix proteins M, which are involved in the envelope and have little sequence relatedness with other paramyxoviruses, as well as M2-1 and M2-1 (Valarcher and Taylor, 2007). There are also two other non-structural or accessory proteins, NS1 and NS2, that may also affect RNA synthesis, thereby reducing the accumulation of unencapsidated genomes and dsRNA as one mechanism of their recognized inhibition of induction of type I interferons (Collins and Melero, 2011).

10.3 Clinical Disease and Diagnosis

Clinical disease associated with bRSV infection most often occurs in calves from 2 to 6 months of age (van der Poel *et al.*, 1994; Baker *et al.*, 1992), although fatal disease can certainly occur in adult animals that are naïve or have weak immunity (Ellis *et al.*, 1996). This is similar to the situation with hRSV infection in humans (van der Poel *et al.*, 1994). Arguably, the most affected calves are those that are not immunologically primed by endemic infection in the herd while they have disease-sparing concentrations of maternal antibodies, although this is difficult to confirm. From a clinical standpoint, a diagnostic feature of uncomplicated acute bRSV infection that approaches pathognomonicity and differentiates it from other calfhood pneumonia is the abrupt onset of tachypnoea and expiratory dyspnoea or 'puffing' in affected animals. Tachypnoea can be >100 breaths/minute and is often accompanied by cervical extension and open-mouthed breathing as the disease progresses. These signs together with pyrexia (≥40°C), anorexia and variable coughing and nasal discharge can begin as early as 3 days after experimental infection with low-dose aerosolized bRSV (West *et al.*, 1999) that closely mimics the clinical disease and lesions reported in field cases (Baker *et al.*, 1992).

From a laboratory perspective, there are numerous approaches and techniques for obtaining a diagnosis of active or previous infection with bRSV, ante-mortem, and confirmatory of bRSV-related disease post-mortem. From the time of its initial discovery, virus isolation has been used to detect the agent in actively infected animals. Bovine respiratory syncytial virus grows in a range of cell types, including Madin-Darby bovine kidney cells, Vero cells and embryonic bovine tracheal cells. Typical cytopathic effect involving the formation of large syncytia is suggestive of bRSV infection, which can be confirmed and differentiated from bPI3 using immunofluorescence (Rossi and Kiesel, 1977) or immunohistochemistry (West *et al.*, 1999). The success of isolation may be improved by the use of rayon-tipped swabs and transport medium-contained magnesium salts; however, this has not been formally examined. Inclusion of magnesium salts may interfere with other testing, such as PCR assays.

bRSV is labile and virus isolation attempts are often thwarted by this lability, which has been recognized since bRSV was first isolated (Smith *et al.*, 1975). It is also highly membrane bound and can be of low titre, which can further complicate isolation attempts (Baker *et al.*, 1992; Collins and Melero, 2011). Because of these problems, and the time-consuming nature of virus isolation, other antigen detection methods were developed. Immunofluorescent staining of clinical material has been used (Thomas and Stott, 1981; Kimman *et al.*, 1986), but is often plagued by background and non-specific staining, resulting in low specificity and sensitivity. Factors contributory to this include autolysis, difficulty in determining the (cellular) identity of stained material, stability of the sample and quenching of the fluorescent signal over time. To overcome these problems several other antigen/virus detection methods have been developed and tested. An antigen capture enzyme immunoassay (EIA) targeting the N protein was shown to be sensitive, specific and cross-reactive among several bRSV isolates. In addition, based on the high degree of conservation between some hRSV and bRSV proteins, notably N and F, EIAs developed for hRSV have been successfully applied to clinical samples from bRSV-infected cattle, as a means of rapid diagnosis (Osorio *et al.*, 1989). Testing results with EIAs, either bRSV-specific or heterologous, compare favourably with virus isolation and PCR, although EIAs are probably somewhat less sensitive than the latter two techniques. Nevertheless, this technology has not been applied in diagnostic laboratories.

Several reverse transcription polymerase chain reactions (RT-PCR) targeting conserved sequences in glycoprotein (Vilcek *et al.*, 1994), fusion protein (Oberst *et al.*, 1993; Vilcek *et al.*, 1994; Larsen *et al.*, 1999) and nucleoprotein (Valarcher *et al.*, 1999) genes have been developed and tested in individual research laboratories. More recently real time RT-PCRs have been developed for the F gene (Achenbach *et al.*, 2004; Hakhverdyan *et al.*, 2005) and the N gene (Boxus *et al.*, 2005; Willoughby *et al.*, 2008), with reported sensitivities of 171 copies, 3.16 $TCID_{50}$/ml, 1000 copies and 10 copies, respectively, adding a further dimension of sensitivity in detection. Although the PCR tests have generally shown good sensitivity and specificity when applied to clinical specimens, they suffer the problem of all PCR tests: difficulty in standardization because of differences in target choice, differences in suppliers of reagents, thermocyclers, laboratory procedures and technician skill. The availability of commercial tests (Timsit *et al.*, 2010) has the potential for standardization, so the results among laboratories can be better compared. In addition to improved sensitivity and specificity, advantages of using EIA or RT-PCR over isolation are that nasopharyngeal swabs can be handled and transported under more normal field conditions (overnight ground transport on ice packs) without apparent loss of sensitivity of detection, and that these techniques are not susceptible to the virus neutralization or blocking of antigen binding by mucosal antibody that develops progressively within days of infection. As with virus isolation and EIA, a potential limitation of PCR testing is the timing of sampling relative to exposure to the virus. Primarily based on experimental infections with low-dose aerosolized bRSV, it has been demonstrated that virus replication peaks approximately 4–5 days after exposure and that the presence of virus as measured by RT-PCR and/or virus isolation in readily accessible clinical specimens (nasopharyngeal swabs) declines precipitously thereafter (West *et al.*, 1998, 2000; Ellis *et al.*, 2001). It may reach undetectable levels in many calves by 8 days after infection (West *et al.*, 1998), although bRSV has been detected by real time RT-PCR up to 20 days after intranasal vaccination in calves (Timsit *et al.*, 2009).

As mentioned, the detection of antibody as a means of documenting previous infection was essential in the original implication of bRSV in respiratory disease in cattle (Doggett *et al.*, 1968). With the advent of PCR, and, to a lesser extent, EIA, serological testing has been superseded by rapid virus/antigen detection methods, notably PCR, in many laboratories. Nevertheless if virus/

antigen detection fails due to the timing of sampling or technical issues, testing of acute and convalescent (7–10 days after acute sample) can be very useful in implicating bRSV in an outbreak of respiratory disease. In addition to the originally described and still used virus neutralization (VN) test, other serologic assays that have been used to detect antibodies to bRSV include complement fixation (Gillete, 1983), enzyme-linked immunosorbant assay (ELISA; Gillete, 1983; West and Ellis, 1997), competitive (blocking) ELISA specific for the F protein (Rhodes *et al.*, 1988) and isotype (IgM)-specific ELISA (Westenbrink and Kimman, 1987). In addition, a variant on the neutralization test is a microneutralization ELISA (Ellis *et al.*, 1995), which combines the ability to detect functional (neutralizing) antibody with a more quantitative, less subjective, outcome measure, computerized optical density values. This test also addresses the potential disparity between results in VN versus ELISA testing that some have reported (Gillete, 1983). An advantage of the isotype-specific IgM ELISA over the standard ELISA that detects IgG is that it can potentially differentiate recent versus past exposure to the virus, and is therefore useful in implicating bRSV in outbreak situations (Westenbrink and Kimman, 1987). Inclusion of bRSV antigen into multiplex ELISAs has yet to improve on, or equal, the results with single component antibody detection tests (Anderson *et al.*, 2011).

Gross pulmonary lesions consisting of locally extensive, depressed plum-coloured areas, atelectasis and consolidation cranioventrally, and hyperinflation with interlobular emphysema and formation of bullae dorsally, although not pathognomonic, are highly suggestive of bRSV-associated pneumonia at post-mortem (Fig. 10.1). Confirmation of bRSV infection in such lesions was first accomplished by immunofluorescence staining of frozen sections (Thomas and Stott, 1981). Although this approach is fast and inexpensive, it is complicated by non-specific reactions and problems in interpretation, including the actual cellular location of the antigen. This technique has been largely replaced by immunohistochemical (IHC) staining using polyclonal sera or monoclonal antibodies raised against bRSV or hRSV (Fig. 10.2; Haines *et al.*, 1989), or *in situ* hybridization (ISH; Viuff *et al.*, 1996) of lung lesions. Enzyme immunoassays and various RT-PCR and real time RT-PCR have also been applied to lung tissue at post-mortem. However, in contrast to IHC and ISH, these techniques do not allow a direct association of the virus with lesions. Moreover, the high sensitivity of PCR tests may detect subclinical infections and possible persistent infections, which have been proposed (van der Poel *et al.*, 1993; De Jong *et al.*, 1996) but not well documented, neither of which may be primary or significant in the terminal illness. Regardless of the test used post-mortem, as with virus isolation and other techniques ante-mortem, there can be false negatives if the testing is done too long after bRSV infection. This can be especially problematic in feedlot, or other cattle that die or are euthanized due to BRDC following one or more treatments (Haines *et al.*, 1989). Fixation in formalin for more than a few days can mask bRSV antigen, which can be unmasked by various retrieval methods (White *et al.*, 1998). However, the virus may not be detectable in lung lesions by immunohistochemistry

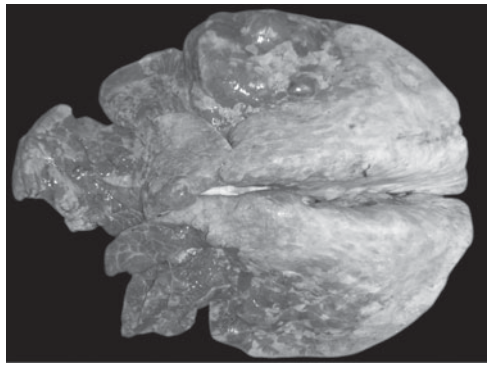


Fig. 10.1. Gross pulmonary pathology in a 3-month-old calf 6 days after infection with bRSV. Note depressed dark cranioventral areas (atelectasis and consolidation) and hyperinflated cuadodorsal areas with interlobular emphysema and formation of bullae.

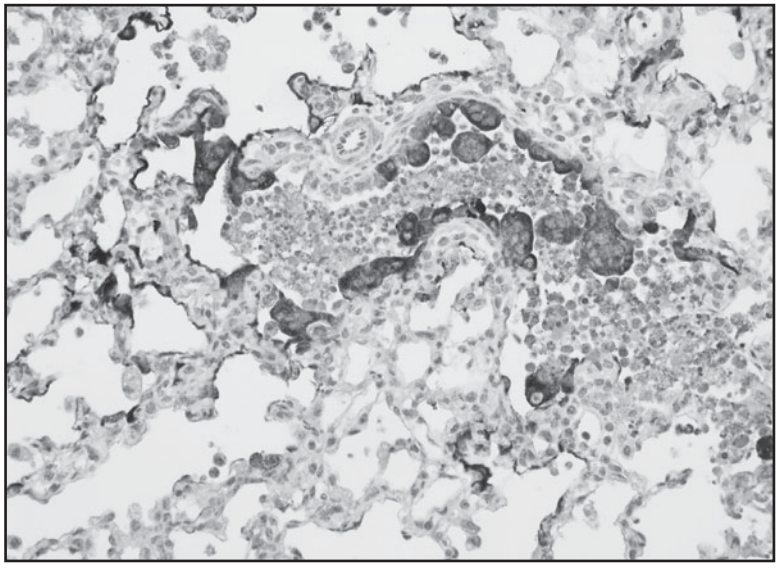


Fig. 10.2. Pulmonary histopathology in a 3-month-old calf 6 days after infection with bRSV. Immunohistochemical staining demonstrates copious bRSV antigen (black material) in bronchiolar cells, syncytial cells and pneumocytes lining alveoli.

in even severely affected calves by as early as 8 days after experimental infection (West *et al.*, 1999; J.A. Ellis, unpublished observations, 2012).

In summary, as in virtually all cases of infectious disease, the diagnosis of bRSV as a causative role in respiratory disease in cattle involves a combination of history, clinical findings, necropsy and laboratory testing. History of an outbreak of respiratory disease in 2–3-month-old or older calves, at a time when passive immunity is waning or has waned, together with signs of fever, tachypnoea and expiratory dyspnoea would be suggestive of bRSV infection. This infection could be confirmed, ante-mortem, with virus isolation from nasopharyngeal swabs, paying attention to handling and transport, or another virus/antigen detection method. Post-mortem, typical gross pulmonary lesions together with visualization of bRSV in typical histological lesions, necrotizing bronchiolitis/alveolitis, would be definitive. Paired serum samples for the detection of bRSV antibodies can be useful, especially in situations where sampling of clinical specimens or necropsy material was not done, or was done much beyond a week after the onset of clinical signs.

10.4 Pathogenesis: From Cells to Whole Animals

Following transmission, by contact with nasal secretions or aerosol over short distances, bRSV can be found in a variety of ciliated and non-ciliated epithelial cells in the respiratory tract, including the airways and pulmonary parenchyma (Castleman *et al.*, 1985; Viuff *et al.*, 1996, 2002). The role of fomite transmission, as occurs in hRSV (Hall, 2000), is undocumented in cattle, but very likely to occur, especially in confinement-reared calves. Viral attachment and entry into cells is initiated by loose binding of the G protein with membrane glycosamino glycans (GAG), notably heparin moieties, probably together with an interaction between other cellular proteins and the F protein. This is followed by cleavage of the F protein into two subunits, F1 and F2. High affinity, species-specific binding of the F2 subunit to an unidentified receptor allows viral penetration into the

cytoplasm (Schlender *et al.*, 2003). There is scant evidence of significant infection of any cells beyond respiratory epithelium by bRSV *in vivo* (Viuff *et al.*, 2002). In addition, in contrast to the other recognized paramyxovirus that is causally associated with respiratory disease in cattle, bovine parainfluenza-3 (bPI3), it is unlikely that bRSV productively infects pulmonary alveolar macrophages (PAM) *in vitro* or *in vivo* (Schrijver *et al.*, 1995; Viuff *et al.*, 2002). bRSV antigen present in PAM is therefore most likely phagocytized material from virally damaged infected epithelial cells (Viuff *et al.*, 2002). Studies *in vitro* (Goris *et al.*, 2009) failed to demonstrate bRSV infection in cultured differentiated ciliated bovine airway cells, in contrast to bPI3, and suggested that environmental or physiologic stimuli *in vivo*, maybe surfactant (Harris and Werling, 2003), render target cells susceptible to bRSV infection. After the cytoplasmic RNA replication and transcription and translation of viral mRNA, nucleocapsids form in the cytoplasm and migrate with the M protein to the cellular membrane in which viral glyoproteins F and G are embedded. Viral particles then bud directly through the apical membrane. In polarized human cells infected with hRSV and probably bRSV-infected bovine cells (Valarcher and Taylor, 2007), this budding occurs without obvious cytopathology, so how do the characteristic and extensive lesions associated with bRSV infection occur? Studies *in vivo* (Viuff *et al.*, 2002) and *in vitro* (Michel *et al.*, 2008) indicate the primary role of death of infected cells is by apoptotic mechanisms, with progressive loss of cells in the upper to lower airways, and then in the pulmonary parenchyma (type I and type II pneumocytes). There is a 'trade-off' between causing death of the host cell and prolonging its survival as a site for virus production. Studies with hRSV document virus-mediated triggering of anti-apoptotic pathways early in infection (Groskreutz *et al.*, 2007) that are also likely to occur in bRSV-infected cattle. In addition to participating in viral entry, the F2 subunit of the fusion protein mediates syncytium formation, which is a characteristic feature of bRSV-mediated cytopathology, *in vivo* and *in vitro* (Valarcher and Taylor, 2007). The result of another post-translational modification of the fusion protein is the generation of virokinin (Zimmer *et al.*, 2003), which induces smooth muscle contraction and may contribute to bronchoconstriction and clinical respiratory disease, although this mechanism remains to be better documented *in vivo*. Together with other *in vitro* studies with hRSV (Zhang *et al.*, 2002; Wright *et al.*, 2005), available data indicate that RSVs are not inherently highly cytopathic, aside from their impairment of ciliary beating, and that host factors are responsible for much of the disease associated with infection by these agents.

The course and severity of infectious disease at the whole animal level is determined by some combination of agent and host factors, both of which are poorly understood in the case of bRSV infection. As with any single-stranded RNA virus, the error-prone viral polymerase (RNA-dependent RNA polymerase; L gene product) together with the lack of exonuclease proofreading affects the quasi-specific nature of bRSV and other pneumoviruses. The well-recognized, but poorly documented empirical observation that *in vitro* vs *in vivo* propagation of bRSV leads to rapid attenuation of the virus (West *et al.*, 1999) is evidentiary of variation within the quasi-species of a single isolate, and how microenvironmental selection pressure can result in the survival or predominance of different variants within the population. Beyond that, genetic and antigenic variation in bRSV populations is predictable and documented. For example, variation of up to 11%, mostly in the G protein, has been reported in sequential isolates from outbreaks in the same herd (Larsen *et al.*, 2000). The biological significance of inter-isolate variation with regard to the propensity for reinfection, differential virulence and antibody escape mutation is debated (Larsen *et al.*, 2000; Deplanche *et al.*, 2007; Valarcher and Taylor, 2007). How expected variation in other gene products, such as NS1 and NS2 that modulate host innate immune responses, may affect pathogenesis is poorly

understood in both bRSV and better studied hRSV infections (Valarcher and Taylor, 2007; Collins and Melero, 2011).

From the standpoint of host factors, one of the most controversial and unresolved issues in RSV pathobiology is the role of the host immune response, both innate and acquired, in disease versus protection. Historically and currently, much of the thinking in this area has been based on voluminous studies of laboratory rodents, primarily mice, infected with hRSV, and also more recently bRSV (Spilki *et al.*, 2006) that focus on immunopathologic mechanisms of disease. Unfortunately, these studies uniformly disregard the obvious deficiencies of the rodent models (Power, 2008), including, rare if any clinical disease, the failure to document significant viral replication after inoculation of large amounts of cultured hRSV, and the absence of gross and histologic lesions that are similar to those found in either bRSV-infected cattle (Viuff *et al.*, 2002) or HRSV-infected humans (Johnson *et al.*, 2007), in other words that RSV infection in rodents is, at most, abortive. In addition to these deficiencies, rodent models of RSV infection overlook the potential role of species differences in immune responses, such as a predominance of γ/δ T cells in young ruminants versus rodents (Hein and Mackay, 1991). A dichotomized Th1/Th2 response to RSV, with Th2 being pathogenic, is the central dogma of immunopathogenesis in mouse models of the hRSV infection (Openshaw and Tregoning, 2005). Most of the few data available in cattle (West *et al.*, 2000; Ellis *et al.*, 2001; Woolums *et al.*, 2004; Antonis *et al.*, 2006) and conflicting data in humans (Collins and Melero, 2011) indicate that responses to RSVs, including inactivated vaccinal antigens, in the natural target species are more complicated, and that a strict Th1 versus Th2 bias is not particularly relevant to understanding pathogenic versus protective immune responses in cattle or humans. There are also significant species-specific differences in inflammatory responses, such as the differential predominance and effects of chemokines (Widdison and Coffey, 2011). For example, the chemokines RANTES and MIP-Iα are potent eosinophil chemoattractants in humans and correlated to more severe hRSV-associated lower airway disease in human infants (Harrison *et al.*, 1999), but increased transcription of mRNA for these chemokines was not found in RSV-infected cattle (Valarcher *et al.*, 2006). Mechanisms involved in species restriction of RSV infection are incompletely understood. In addition to the species-specific nature of the initial interaction between RSV and the host cell mediated by the F2 subunit of the F protein (Schlender *et al.*, 2003), the abortive nature of RSV infections in rodents may be related to the inability of hRSV and bRSV, NS1 and/or NS2 proteins to interfere with the activation of the IRF-3 and other pathways involved in the transcription of type 1 interferon genes in mice and other rodents (Schlender *et al.*, 2000). Because of this in species such as mice and cotton rats, innate immune responses may clear the virus early in infection, and no disease results. Species differences in the anatomy and physiology of the respiratory tract may also contribute to the manifestations and severity of disease in the natural target host. It has long been recognized that cattle have lungs that are relatively undersized for their body mass (Veit and Farrell, 1978; Robinson, 1982). Relative to mice, their airways are of small diameter and have many submucosal glands (Veit and Farrell, 1978; Irvin and Bates, 2003). Cattle, and to a lesser extent humans, when compared with species such as mice and dogs, also have reduced collateral ventilation in the lung, which predisposes them to the development of emphysema in responses to certain pulmonary injuries, such as the partial occlusion of small airways subsequent to the typical bronchiolytic changes characteristic of bRSV infection (Veit and Farrell, 1978; Robinson, 1982; Irvin and Bates, 2003). Beyond the lung, cattle have a large fermentation vat, the rumen, which is capable of absorbing and producing pneumotoxicants, which can then be delivered to the lung in the systemic circulation. In fact, a synergism between the cytopathological effects of bRSV infection and rumen-derived pneumotoxicants, as represented by 3-methylindole, has been demonstrated

experimentally (Bingham *et al.*, 1999). The importance of this interaction in the context of nutritional changes and altered rumen metabolism in cattle, and their variable susceptibility to BRD, remain to be determined. Therefore, although the findings in the mouse model have been widely and simplistically extrapolated to cattle and human RSV infections (Openshaw and Tregoning, 2005; Valarcher and Taylor, 2007; Gershwin, 2008), these studies, arguably, are largely irrelevant, or at least potentially confusing to the biology in cattle (and humans), and will not be further addressed here.

Age is a recognized risk factor to the development of disease following RSV infection in both calves and human infants (van der Poel *et al.*, 1994; Collins and Melero, 2011). Historically, the disease-sparing effect of maternal antibodies early in life, followed by the decay of this passive immunity, has been implicated in the age-specific appearance of a 'window of susceptibility' to developing clinical infections in calves (Bryson *et al.*, 1978; Kimman *et al.*, 1988). This is supported by the epidemiology of infection in cattle populations (Baker *et al.*, 1992; van der Poel *et al.*, 1993), data from experimental infections of seropositive calves (Belknap *et al.*, 1991) and currently held immunological principles concerning the disease-sparing effect of maternal antibodies. More recent studies have addressed the possible association between age-specific involvement of innate immune responses and disease severity (Grell *et al.*, 2005; Antonis *et al.*, 2010), focusing on the observation that very young neonatal calves and older calves (>6 months) have an apparent reduced susceptibility to disease following infection. After experimental infection with bRSV, circulating blood leukocytes from 1–5-week-old colostrum-fed calves, compared to those from older (9–16- or 32–37-week-old) infected calves, contained and secreted higher amounts of the proinflammatory cytokine TNFα as well as IFNγ (Grell *et al.*, 2005). The older calves had higher levels of circulating B cells and accompanying, apparently disease-sparing, antibody response after infection. Confirming and extending these findings, Antonis and colleagues (2010) documented higher concentrations of TNFα in lung lavage fluid following bRSV infection of seronegative gnotobiotic 6-week-old calves versus 1-day-old seronegative gnotobiotic calves. This was associated with significantly less disease in the neonatal calves. Together, these findings suggest that there may be a bimodal innate response, involving at least TNFα, to bRSV infection in cattle, with neonatal and older calves having less pronounced inflammatory responses and concordantly less disease. Further work is required to better understand the contribution of the age-dependent interaction between the virus and the bovine innate/inflammatory response. A histologic feature of the response to naturally acquired (Bryson *et al.*, 1978; Baker *et al.*, 1992) and experimental bRSV infections is a mixed inflammatory cell infiltrate in airways, comprising primarily neutrophils and monocyte/macrophages (Castleman *et al.*, 1985; Viuff *et al.*, 2002). It is currently unclear which endogenous chemoattracts are responsible for this influx. Transcripts for RANTES, MIP-1α, MIP-2 α, MIP-3α and MCP-2 were detected in bovine blood-derived dendritic cells following stimulation with bRSV *in vitro* (Werling *et al.*, 2002). However, as mentioned, transcripts for RANTES and MIP-1α were not demonstrable *in vivo* following infection of cattle with bRSV (Valacher *et al.*, 2006). Interleukin-8 is another potent chemoattractant for neutrophils in cattle, and although high quantities of transcript expression were demonstrable in bacterial (*Mannheimia haemolytica*) pneumonia (Czuprynski *et al.*, 2004), mRNA for IL-8 was not above steady state levels in cattle experimentally infected with bRSV (Caswell *et al.*, 1998). It is possible, although not extensively examined, that C5a resulting from complement fixation by bRSV-infected cells (Kimman *et al.*, 1989) could be the primary chemoattractant for influx of inflammatory cells into bRSV-infected airways and parenchyma. Although RSVs may induce robust inflammatory responses in their natural hosts, it is not yet clear whether this is primarily protective or pathogenic (Collins and Melero, 2011).

It is generally thought, on the basis of epidemiologic studies, examination of diagnostic material and experimental infections, that there is a positive correlation between the extent of viral replication (viral load) and disease severity in bRSV-infected cattle (Haines *et al.*, 1989; Baker *et al.*, 1992; West *et al.*, 1999), and in hRSV-infected humans (Karron *et al.*, 1997; DeVincenzo *et al.*, 2005). Further supportive of this is the consistent association between decreased viral load (shedding) and reduced clinical disease and lesions in bRSV-vaccinated versus unvaccinated calves (West *et al.*, 2000; Ellis *et al.*, 2001, 2010). This reduction in viral shed and disease has been associated with a range of adaptive B and T cell responses. Nevertheless, the observations primarily from aforementioned *in vitro* studies concerning the minimal cytopathology of RSVs, together with work implicating immune responses in pathogenesis of RSV-associated disease, have raised questions about the correlation between viral load and disease in RSV-infected calves (Antonis *et al.*, 2010) and in human pediatric patients (Hall *et al.*, 1976; Wright *et al.*, 2002). In cattle, in one study comparing responses to post-experimental infection in seronegative 1-day-old gnotobiotic calves versus 6-week-old calves, there was significantly more and longer nasal shed of bRSV in the neonates. This increased viral shed was associated with decreased lung pathology in the neonates versus the 6-week-old calves. However, in that study there was only minimal pulmonary pathology in both groups (<5% lung involvement), compared to that found in other models that associated viral shed with increased lung involvement (>40%), making it difficult to draw conclusions about severity of disease. While not discounting the role of the host inflammatory response, certainly in calves, copious bRSV antigen can be consistently identified in viable and dead epithelial target cells in bronchiolytic lesions in the first 5–6 days after infection (Fig. 10.2) and significantly less so thereafter (West *et al.*, 1999, 2000; Viuff *et al.*, 2002). These findings are coincident with viral shedding, so that associations between viral load and pulmonary pathology could be, at least in part, a function of the timing of sampling.

There is evidence that bRSV-infection can predispose to allergic pulmonary disease in response to some antigens, including those of bRSV, when these antigens are given by some routes and in some combinations of exposure (Gershwin *et al.*, 2008). The prevalence of this immunopathological phenomenon and its contribution to BRDC in cattle populations is unclear. The epidemiology of infection and disease in both cattle and humans infected by their respective RSVs is that clinical disease generally becomes markedly less severe with age and repeated exposure, which is inconsistent with allergic responses and/or a predominance of IgE-mediated responses being a significant component of the disease in the vast majority of individuals in the target host populations (van der Poel *et al.*, 1994; Collins and Melero, 2011).

In summary, the pathogenesis of bRSV-associated disease is complicated and poorly understood. It is complicated by the unknown, but likely, contribution of variation in the bRSV quasi-species to virulence, and variation in virulence. Traditionally, most *in vitro* and *in vivo* studies of viral factors have been done using cultured viruses, which produce little disease *in vivo*, further complicating the picture. The RSVs infect, and most often cause disease, at an immunologically complicated time in the young lives of their natural hosts; a time of transition from passive immunity to active immunity. Relatedly, it is a time when the innate immune system may exhibit a more pronounced (inflammatory) response to infectious agents, further raising questions about the overall role of the immune response in pathogenesis. Currently there are unanswered questions related to the importance and role of viral load (and cytopathology) versus host response in the development of disease. Applying Occam's razor to this question, it may be as simple as more and uncontrolled viral replication due to poor (acquired) immunological control of virus infection leads to more inflammation and more clinical disease. Hopefully, time will tell.

References

Achenbach, J.E., Topliff, C.L., Vassilev, V.B., Donis, R.O., Eskridge, K.M. and Kelling, C.L. (2006) Detection and quantitation of bovine respiratory syncytial virus using real time quantitative RT-PCR and quantitative competitive RT-PCR assays. *Journal of Virological Methods* 121, 1–6.

Anderson, S., Wakeley, P., Wibberley, G., Webster, K. and Sawyer, J. (2011) Development and evaluation of a Luminex multiplex serology assay to detect antibodies to bovine herpes virus 1, parainfluenza 3 virus, bovine viral diarrhoea virus, and bovine respiratory syncytial virus, with comparison to existing ELISA detection methods. *Journal of Immunological Methods* 366, 79–88.

Antonis, A.F., Claassen, E.A., Hensen, E.J., de Groot, R.J., de Groot-Mijnes, J.D., Schrijver, R.S. and van der Most, R.G. (2006) Kinetics of antiviral CD8 T cell responses during primary and post-vaccination secondary bovine respiratory syncytial virus infection. *Vaccine* 24, 1551–1561.

Antonis, A.F, de Jong, M.C., van der Poel, W.H., van der Most, R.G., Stockhofe-Zurwieden, N., Kimman, T. and Schrijver, R.S. (2010) Age-dependent differences in the pathogenesis of bovine respiratory syncytial virus infections related to the development of natural immunocompetence. *Journal of General Virology* 91, 2497–2506.

Baker, J.C., Wilson, E.G., McKay, G.L., Stanek, R.J., Underwood, W.J., Vellcer, L.F. and Mufson M.A. (1992) Identification of subgroups of bovine respiratory syncytial virus. *Journal of Clinical Microbiology* 30, 1120–1126.

Belknap, E., Baker, J.C., Patterson, J.S., Walker, R.D., Haines, D.M. and Clark, E.G. (1991) The role of passive immunity in bovine respiratory syncytial virus-infected calves. *Journal of Infectious Diseases* 163, 470–476.

Bingham, H.R., Morley, P.S., Wittum, T.E., Bray, T.M., West, K.H., Slemons, R.D., Ellis, J.A., Haines, D.M., Levy, M.A., Sarver, C.F., Saville, W.J. and Cortese, V.S. (1999) Synergistic effects of concurrent challenge with bovine respiratory syncytial virus and 3-methylindole. *American Journal of Veterinary Research* 60, 563–570.

Boxus, M., Letellier, C. and Kerkhofs, P. (2005) Real time RT-PCR for the detection and quantitation of bovine respiratory syncytial virus. *Journal of Virological Methods* 125, 125–130.

Brodersen, B.W. (2010) Bovine respiratory syncytial virus. *Veterinary Clinics of North America: Food Animal Practice* 26, 323–333.

Bryson, D.G., McFerran, J.B., Ball, H.J. and Neill, S.D. (1978) Observations on outbreaks of respiratory disease in housed calves – (2) pathological and microbiological findings. *Veterinary Record* 103, 503–509.

Bukreyev, A., Yang, L., Fricke, J., Cheng, L., Ward, J.M., Murphy, B.R. and Collins, P.L. (2008) The secreted form of respiratory syncytial virus G glycoprotein helps the virus evade antibody-mediated restriction of replication by acting as an antigen decoy and through effects on Fc receptor-bearing leukocytes. *Journal of Virology* 82, 12,191–12,204.

Castleman, W.L., Chandler, S.K. and Slauson, D.O. (1985) Experimental bovine respiratory syncytial virus infection in conventional calves: ultrastructural respiratory lesions. *American Journal of Veterinary Research* 46, 554–560.

Caswell, J.L., Middleton, D.M., Sorden, S.D. and Gordon, J.R. (1998) Expression of the neutrophil chemoattractant interleukin-8 in the lesions of bovine pneumonic pasteurellosis. *Veterinary Pathology* 35, 124–131.

Chanock, R. and Finberg, L. (1957) Recovery from infants with respiratory illness of virus related to chimpanzee coryza agent (CCA) II. Epidemiologic aspects of infection in infants and young children. *American Journal of Hygiene* 66, 291–300.

Chanock, R., Roizman, B. and Myers, R. (1957) Recovery from infants with respiratory illness of virus related to chimpanzee coryza agent (CCA). I. Isolation properties and characterization. *American Journal of Hygiene* 66, 281–290.

Collins, P.L. and Melero, J.A. (2011) Progress in understanding and controlling respiratory syncytial virus: still crazy after all these years. *Virus Research* 162, 80–99.

Czuprynski, C.J., Leite, F., Sylte, M., Kuckleburg, C., Schultz, R., Inzana, T., Behling-Kelly, E. and Corbeil, L. (2004) Complexities of the pathogenesis of *Mannheimia haemolytica* and *Haemophilus somnus* infections: challenges and potential opportunities for prevention? *Animal Health Research Reviews* 5, 277–282.

De Jong, M.C.M., Van der Poel, W.H.M., Kramps, J.A., Brand, A. and van Oirschot, J.T. (1996) Quantitative investigation of population persistence and recurrent outbreaks of bovine respiratory syncytial virus on dairy farms. *American Journal of Veterinary Research* 57, 628–633.

Deplanche, M., Lemaire, M., Mirandette, C., Bonnet, M., Schelcher, F. and Meyer, G. (2007) In vivo evidence for quasispecies distributions in the bovine respiratory syncytial virus genome. *Journal of General Virology* 88, 1260–1265.

DeVincenzo, J.P., El Saleeby, C.M. and Bush, A.J. (2005) Respiratory syncytial virus load predicts disease severity in previously healthy infants. *Journal of Infectious Diseases* 191, 1861–1868.

Doggett, J.E., Taylor-Robinson, D. and Gallop, R.G.C. (1968) A study of an inhibitor in bovine serum active against respiratory syncytial virus. *Archives Gesamte Virusforschung* 23, 126–137.

Ellis, J.A., Hassard, L. and Morley, P.S. (1995) Development and application of a microneutralization ELISA for the detection of antibodies to bovine respiratory syncytial viruses. *Journal of Veterinary Diagnostic Investigation* 7, 183–189.

Ellis, J.A., Philibert, H., West, K., Clark, E., Martin, K. and Haines, D. (1996) Fatal pneumonia in adult dairy cattle associated with active bovine respiratory syncytial virus infection. *Canadian Veterinary Journal* 37, 103–104.

Ellis, J., West, K., Konoby, C., Leard, T., Gallo, G., Conlon, J. and Fitzgerald, N. (2001) Efficacy of an inactivated respiratory syncytial virus vaccine in calves. *Journal of the American Veterinary Medical Association* 218, 1973–1980.

Ellis, J.A., Gow, S.P. and Goji, N. (2010) Response to experimentally induced infection with bovine respiratory syncytial virus following intranasal vaccination of seropositive and seronegative calves. *Journal of the American Veterinary Medical Association* 236, 991–999.

Gershwin, L.J. (2008) Bovine respiratory syncytial virus infection: immunopathologic mechanisms. *Animal Health Research Reviews* 8, 207–213.

Gershwin, L.J., Gunther, R.A., Hornof, W.J. and Larson, R.F. (2008) Effect of infection with bovine respiratory syncytial virus on pulmonary clearance of an inhaled antigen in calves. *American Journal of Veterinary Research* 69, 416–422.

Gillete, K.G. (1983) Enzyme-linked immunosorbent assay use to monitor serum antibodies to bovine respiratory syncytial virus: comparison with complement fixation and neutralization tests. *American Journal of Veterinary Research* 44, 2251–2255.

Goris, K., Uhlenbeck, S., Schwegmann-Wessels, C., Köhl, W., Niedorf, F., Stern, M., Hewicker-Trautwein, M., Bals, R., Taylor, G., Braun, A., Bicker, G., Kietzmann, M. and Herrler, G. (2009) Differential sensitivity of differentiated epithelial cells to respiratory viruses reveals different viral strategies of host infection. *Journal of Virology* 83, 1962–1968.

Grell, S.N., Riber, U., Tjornehoj, K., Larsen, L.E. and Heegaard, P.M. (2005) Age-dependent differences in cytokine and antibody responses after experimental RSV infection in a bovine model. *Vaccine* 23, 3412–3423.

Groskreutz, D.J., Monick, M.M., Yarovinsky, T.O., Powers, L.S., Quelle, D.E., Varga, S.M., Look, D.C. and Hunninghake, G.W. (2007) Respiratory syncytial virus decreases p53 to prolong survival of airway epithelial cells. *Journal of Immunology* 179, 2741–2747.

Haines, D.M., Clark, E.G. and Chelack, B.J. (1989) The detection of bovine respiratory syncytial virus in formalin fixed bovine lung with commercially available monoclonal antibodies and avidin biotin complex immunohistochemistry. *Canadian Journal of Veterinary Research* 53, 366–368.

Hakhverdyan, M., Hagglund, S., Larsen, L.E. and Belák, S. (2005) Evaluation of a single-tube fluorogenic RT-PCR assay for detection of bovine respiratory syncytial virus in clinical samples. *Journal of Virological Methods* 123, 195–202.

Hall, C.B. (2000) Nosocomial respiratory syncytial virus infections: the 'Cold War' has not ended. *Clinical Infectious Diseases* 31, 590–596.

Hall, C.B., Douglas, R.G. Jr and Geiman, J.M. (1976) Respiratory syncytial virus infections in infants: quantitation and duration of shedding. *Journal of Pediatrics* 89, 11–15.

Hall, C.B., Weinberg, G.A., Iwane, M.K., Edwards, K.M., Staat, M.A., Auinger, P., Griffin, M.R., Poehling, K.A., Erdman, D., Grijalva, C.G., Zhu, Y. and Szilagyi, P. (2009) The burden of respiratory syncytial virus infection in young children. *New England Journal of Medicine* 360, 588–598.

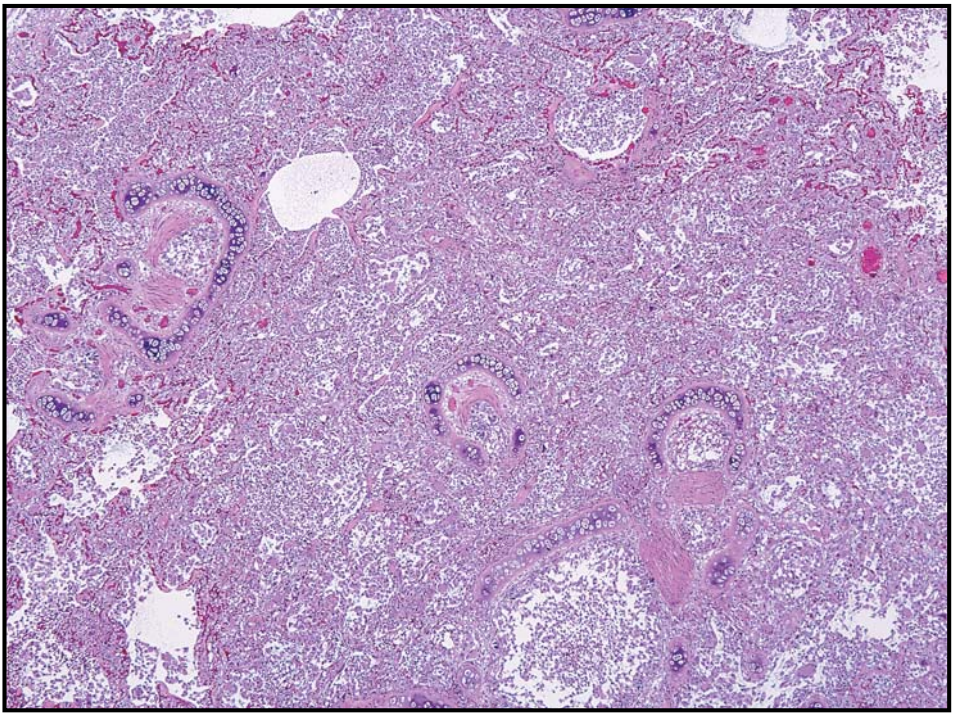
Harris, J. and Werling, D. (2003) Binding and entry of respiratory syncytial virus into host cells and initiation of the innate immune response. *Cellular Microbiology* 5, 671–680.

Harrison, A.M., Bonville, C.A., Rosenberg, H.F. and Domachowske, J.B. (1999) Respiratory syncytial virus-induced chemokine expression in the lower airways: eosinophil recruitment and degranulation. *American Journal of Respiratory Critical Care Medicine* 159, 1918–1924.

Hein, W.R. and Mackay, C.R. (1991) Prominence of γδ T cells in the ruminant immune system. *Immunology Today* 12, 30–34.

Inaba, Y., Tanaka, Y., Sato, K., Ito, H. and Omori, T. (1970a) Nomi virus, a virus isolated from an apparently new epizootic respiratory disease of cattle. *Japanese Journal of Microbiology* 14, 246–248.

1



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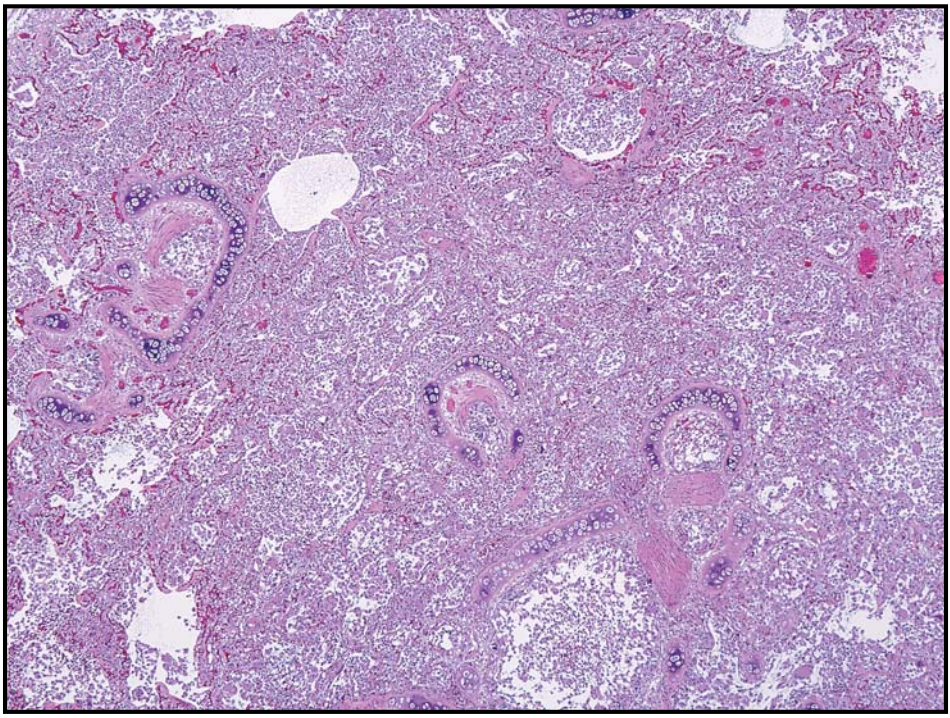

Plate 1. Lung, Mediterranean striped dolphin from the 2006–2007 epizootic. The lung shows intense pneumonic lesions, with characteristic syncytial cells. H&E staining.

Plate 2. Lung, Mediterranean striped dolphin from the 2006–2007 epizootic. Same lung as in Plate 1, showing intense CeMV immunostaining in inflammatory cells, pneumocytes and syncytial cells. CeMV-IHC.

3

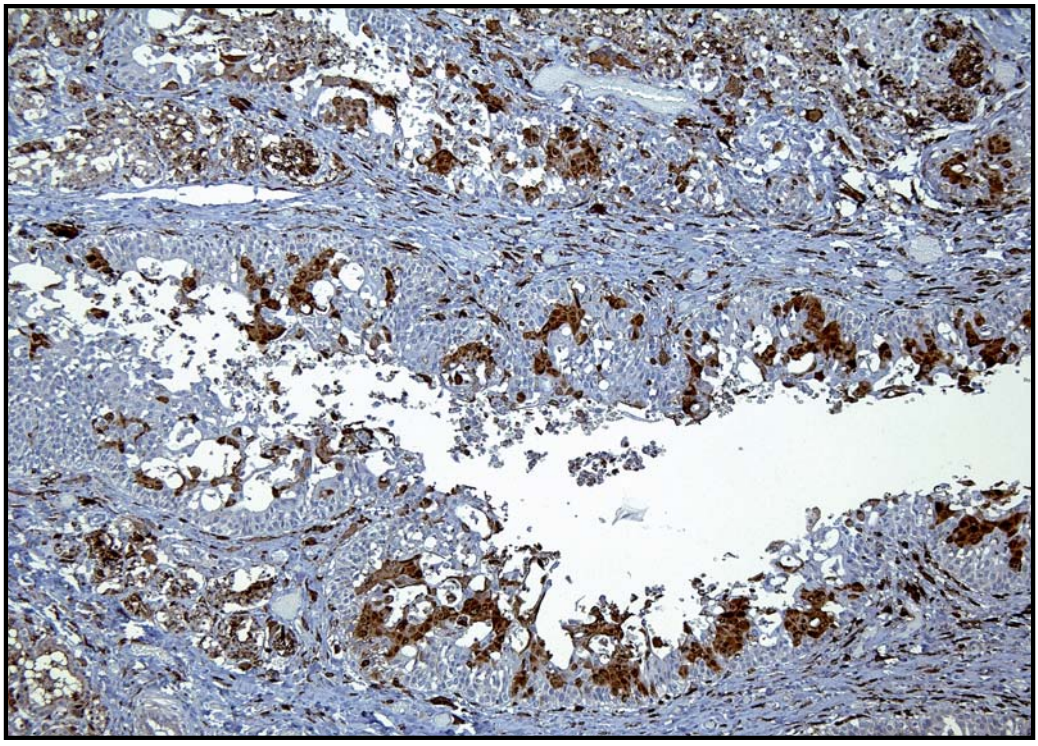

Plate 3. Laryngeal tonsil, Mediterranean striped dolphin from the 2006–2007 epizootic. The epithelial and the lymphoid cells (markedly reduced in number) show intense CeMV immunostaining. CeMV-IHC.

Inaba, Y., Tanaka, Y., Omori, T. and Matumoto, M. (1970b) Isolation of bovine respiratory syncytial virus. *Japanese Journal of Experimental Medicine* 40, 473–474.

Irvin, C.G. and Bates, J.H. (2003) Measuring the lung function in the mouse: the challenge of size. *Respiratory Research* 4, 4–12.

Jacobs, J.W. and Edington, N. (1971) Isolation of respiratory syncytial virus from cattle in Britain. *Veterinary Record* 88, 694–701.

Johnson, J.E., Gonzales, R.A., Olson, S.J., Wright, P.F. and Graham, B.S. (2007) The histopathology of fatal untreated human respiratory syncytial virus infection. *Modern Pathology* 20, 108–119.

Karron, R.A., Wright, P.F., Crowe, J.E. Jr, Clements-Mann, M.L., Thompson, J., Makhene, M., Casey, R. and Murphy, B.R. (1997) Evaluation of two live, cold-passaged, temperature-sensitive respiratory syncytial virus vaccines in chimpanzees and in human adults, infants, and children. *Journal of Infectious Diseases* 176, 1428–1436.

Kimman, T.G., Zimmer, G.M. and Stravers, P.J. (1986) Diagnosis of bovine respiratory syncytial virus infections improved by virus detection in lung lavage samples. *American Journal of Veterinary Research* 47, 143–147.

Kimman, T.G., Zimmer, G.M., Westenbrink, F., Mars, J. and van Leeuwen, E. (1988) Epidemiological study of bovine respiratory syncytial virus infections in calves: influence of maternal antibodies on the outcome of disease. *Veterinary Record* 123, 104–109.

Kimman, T.G., Daha, M.R., Brinkhof, J.M. and Westenbrink, F. (1989) Activation of complement by bovine respiratory syncytial virus-infected cells. *Veterinary Immunology & Immunopathology* 21, 311–325.

Larsen, L.E., Tjornehoj, K., Viuff, B., Jensen, N.E. and Uttenthal, A. (1999) Diagnosis of enzootic pneumonia in Danish cattle: reverse transcriptionpolymerase chain reaction respiratory syncytial virus in naturally and experimentally infected cattle. *Journal of Veterinary Diagnostic Investigation* 11, 416–422.

Larsen, L.E., Tjornehoj, K. and Viuff, B. (2000) Extensive sequence divergence among bovine respiratory syncytial viruses isolated during recurrent outbreaks in closed herds. *Journal of Clinical Microbiology* 38, 4222–4277.

Levine, S., Klaiber-Franco, R. and Paradiso, P.R. (1987) Demonstration that glycoprotein G is the attachment protein of respiratory syncytial virus. *Journal of General Virology* 68, 2521–2524.

Michel, J.-E., Lorek, M., Baxmann, D., Grunwald, T., Keil, G.M. and Zimmer, G. (2008) The fusion protein of respiratory syncytial virus triggers p53-dependent apoptosis. *Journal of Virology* 82, 3236–3249.

Morris, J.A., Blount, R.E. and Savage, R.E. (1956) Recovery of cytopathogenic agent from chimpanzee with coryza. *Proceedings Society of Experimental Biology and Medicin*e 92, 544–549.

Oberst, R.D., Hays, M.P., Hennessy, K.J., Stine, L.C., Evermann, J.F. and Kelling, C.L. (1993) Identifying bovine respiratory syncytial virus by reverse transcription polymerase bovine respiratory syncytial virus by reverse transcriptionpolymerase chain reaction and oligonucleotide hybridizations. *Journal of Clinical Microbiology* 31, 1237–1240.

Openshaw, P.J.M. and Tregoning, J.S. (2005) Immune responses and disease enhancement during respiratory syncytial virus infection. *Clinical Microbiology Reviews* 18, 541–555.

Osorio, F.A., Anderson, G.A., Sanders, J. and Grotelueschen, D. (1989) Detection of bovine respiratory syncytial virus using a heterologous antigen-capture enzyme immunoassay. *Journal of Veterinary Diagnostic Investigation* 1, 210–214.

Paccaud, M.F. and Jacquier, C.I. (1970) A respiratory syncytial virus of bovine origin. *Archives Gesammte Virusforsch* 30, 327–342.

Power, U.F. (2008) Respiratory syncytial virus (RSV) vaccines: two steps back for one leap forward. *Journal of Clinical Virology* 41, 38–44.

Rhodes, M.B., Klucas, C.A., Frey, M.L. and Anderson, G.A. (1988) A blocking ELISA for the detection of specific antibodies to bovine respiratory syncytial virus. *Journal of Veterinary Diagnostic Investigation* 1, 359–369.

Robinson, N.E. (1982) Some functional consequences of species differences in lung anatomy. *Advances in Veterinary Science and Comparative Medicine* 26, 1–33.

Rosenquist, B.D. (1974) Isolation of respiratory syncytial virus from calves with acute respiratory disease. *Journal of Infectious Diseases* 130, 177–182.

Rossi, C.R. and Kiesel, G.K. (1977) Bovine respiratory syncytial virus infection of bovine embryonic lung cultures: a kinetic study by the fluorescent antibody technique. *American Journal of Veterinary Research* 38, 1901–1904.

Schlender, J., Bossert, B., Buchholz, U. and Conzelmann, K.K. (2000) Bovine respiratory syncytial virus nonstructural proteins NS1 and NS2 cooperatively antagonize alpha/beta interferon-induced response. *Journal of Virology* 74, 8234–8242.

Schlender, J., Zimmer, G., Herrler, G. and Conzelmann, K.K. (2003) Respiratory syncytial virus (RSV) fusion protein subunit F2, not attachment protein G, determines specificity of RSV infection. *Journal of Virology* 77, 4609–4616.

Schrijver, R.S., Kramps, J.A., Middel, W.G.J., Langedijk, J.P. and van Oirschot, J.T. (1995) Bovine respiratory syncytial virus replicates minimally in bovine alveolar macrophages. *Archives of Virology* 140, 1905–1917.

Smith, M.H., Frey, M.L. and Dierks, R.E. (1975) Isolation, characterization, and pathogenicity studies of a bovine respiratory syncytial virus. *Archives of Virology* 47, 237–247.

Spilki, F.S., Almeida, R.S., Ferreira, H.L., Gameiro, J., Verinaud, L. and Arns, C.W. (2006) Effects of experimental inoculation of bovine respiratory syncytial virus in different inbred mice lineages: establishment of a murine model for BRSV infection. *Veterinary Microbiology* 118, 161–168.

Tayyari, F., Marchant, D., Moraes, T.J., Duan, W., Mastrangelo, P. and Hegele, R.G. (2011) Identification of nucleolin as a cellular receptor for human respiratory syncytial virus. *Nature Medicine* 17, 1132–1135.

Thomas, L.H. and Stott, E.J. (1981) Diagnosis of respiratory syncytial virus infection in the bovine respiratory tract by immunofluorescence. *Veterinary Record* 108, 432–435.

Timsit, E., Le Drean, E, Maingourd, C., Belloc, C., Guattéo, R., Bareille, N., Seegers, H., Douart, A., Sellal, E. and Assié, S. (2009) Detection by real-time RT-PCR of a bovine respiratory syncytial virus vaccine in calves vaccinated intranasally. *Veterinary Record* 165, 230–233.

Timsit, E., Maingourd, C., Le Drean, E., Belloc, C., Seegers, H., Douart, A. and Assié, S. (2010) Evaluation of a commercial real-time reverse transcription polymerase chain reaction kit for the diagnosis of bovine respiratory syncytial virus infection. *Journal of Veterinary Diagnostic Investigation* 22, 238–241.

Valarcher, J.F. and Taylor, G. (2007) Bovine respiratory syncytial virus infection. *Veterinary Research* 39, 153–180.

Valarcher, J.F., Bourhy, H., Gelfi, J. and Schelcher, F. (1999) Evaluation of a nested reverse transcription-PCR assay based on the nucleoprotein gene for diagnosis of spontaneous and experimental bovine respiratory syncytial virus infections. *Journal of Clinical Microbiology* 37, 1858–1862.

Valarcher, J.F., Furze, J.M., Wyld, S.G., Cook, R., Zimmer, G., Herrler, G. and Taylor, G. (2006) Bovine respiratory syncytial virus lacking the virokinin or with a mutation in furin cleavage site RA(R/K)R109 induces less pulmonary inflammation without impeding the induction of protective immunity in calves. *Journal of General Virology* 87, 1659–1667.

van der Poel, W.H., Kramps, J.A., Middel, W.G., Van Oirschot, J.T. and Brand, A. (1993) Dynamics of bovine respiratory syncytial virus infections: a longitudinal epidemiological study in dairy herds. *Archives of Virology* 133, 309–321.

van der Poel, W.H.M., Brand, A., Kramps, J.A. and Van Oirschot, J.T. (1994) Respiratory syncytial virus infections in human beings and in cattle. *Journal of Infection* 29, 215–228.

Veit, H.P. and Farrell, R.L. (1978) The anatomy and physiology of the bovine respiratory system relating to pulmonary disease. *Cornell Veterinarian* 68, 555–581.

Vilcek, S., Elvander, M., Ballagi-Pordany, A. and Belák, S. (1994) Development of nested PCR assays for detection of bovine respiratory syncytial virus in clinical samples. *Journal of Clinical Microbiology* 32, 2225–2231.

Viuff, B., Uttenthal, A., Tegtmeier, C. and Alexandersen, S. (1996) Sites of replication of bovine respiratory syncytial virus in naturally infected calves as determined by *in situ* hybridisation. *Veterinary Pathology* 33, 383–390.

Viuff, B., Tjornehoj, K., Larsen, L., Røntved, C.M., Uttenthal, A., Rønsholt, L. and Alexandersen, S. (2002) Replication and clearance of respiratory syncytial virus: apoptosis is an important pathway of virus clearance after experimental infection with bovine respiratory syncytial virus. *American Journal of Pathology* 161, 2195–2207.

Wellemans, G., Leunen, J. and Luchsinger, E. (1970) Isolement d'un virus (220/69) serologiquement semblable au virus respiratoire syncytial (RS) humain. *Annals Medicine Veterinaire* 114, 89–93.

Werling, D., Koss, M., Howard, C.J., Taylor, G., Langhans, W. and Hope, J.C. (2002) Role of bovine chemokines produced by dendritic cells in respiratory syncytial virus-induced T cell proliferation. *Veterinary Immunology & Immunopathology* 87, 225–233.

West, K.H. and Ellis, J.A. (1997) Functional analysis of antibody responses of feedlot cattle to bovine respiratory syncytial virus following vaccination with mixed vaccines. *Canadian Journal of Veterinary Research* 61, 28–33.

West, K., Bogdan, J., Hamel, A., Nayar, G., Morley, P.S., Haines, D.M. and Ellis, J.A. (1998) A comparison of diagnostic methods for the detection of bovine respiratory syncytial virus in experimental clinical specimens. *Canadian Journal of Veterinary Research* 62, 245–260.

West, K., Petrie, L., Haines, D.M., Konoby, C., Clark, E.G., Martin, K. and Ellis, J.A. (1999) The effect of formalin-inactivated vaccine on respiratory disease associated with bovine respiratory syncytial virus infection in calves. *Vaccine* 17, 809–820.

West, K., Petrie, L., Konoby, C., Haines, D.M., Cortese, V. and Ellis, J.A. (2000) The efficacy of modified-live bovine respiratory syncytial virus vaccines in experimentally infected calves. *Vaccine* 18, 907–919.

Westenbrink, F. and Kimman, T.G. (1987) Immunoglobulin M-specific enzyme-linked immunosorbent assay for serodiagnosis of bovine respiratory syncytial virus infections. *American Journal of Veterinary Research* 48, 1132–1137.

White, A.K., Hansen-Lardy, L. and Brodersen, B.W. (1998) Enhanced immunohistochemical detection of infectious agents in formalin-fixed, paraffin-embedded tissues following heat-mediated antigen retrieval. *Journal of Veterinary Diagnostic Investigation* 10, 214–217.

Widdison, S. and Coffey, T.J. (2011) Cattle and chemokines: evidence for species-specific evolution of the bovine chemokine system. *Animal Genetics* 42, 341–353.

Willoughby, K., Thomson, K., Maley, M., Gilray, J., Scholes, S., Howie, F., Caldow, G. and Nettleton, P.F. (2008) Development of a real time reverse transcriptase polymerase chain reaction for the detection of bovine respiratory syncytial virus in clinical samples and its comparison with immunohistochemistry and immunofluorescence antibody testing. *Veterinary Microbiology* 126, 264–270.

Woolums, A.R., Gunther, R.A., McArthur-Vaughan, K., Anderson, M.L., Omlor, A., Boyle, G.A., Friebertshauser, K.E., McInturff, P.S. and Gershwin, L.J. (2004) Cytotoxic T lymphocyte activity and cytokine expression in calves vaccinated with formalin-inactivated bovine respiratory syncytial virus prior to challenge. *Comparative Immunology, Microbiology & Infectious Diseases* 27, 57–74.

Wright, P.F., Gruber, W.C., Peters, M., Reed, G., Zhu, Y., Robinson, F., Coleman-Dockery, S. and Graham, B.S. (2002) Illness severity, viral shedding, and antibody responses in infants hospitalized with bronchiolitis caused by respiratory syncytial virus. *Journal of Infectious Diseases* 18, 1011–1018.

Wright, P.F., Ikizler, M.R., Gonzales, R.A., Carroll, K.N., Johnson, J.E. and Werkhaven, J.A. (2005) Growth of respiratory syncytial virus in primary epithelial cells from the human respiratory tract. *Journal of Virology* 79, 8651–8654.

Zhang, L., Peeples, M.E., Boucher, R.C., Collins, P.L. and Pickles, R.J. (2002) Respiratory syncytial virus infection of human airway epithelial cells is polarized, specific to ciliated cells, and without obvious cytopathology. *Journal of Virology* 76, 5654–5666.

Zimmer, G., Rohn, M., McGregor, G.P., Schemann, M., Conzelmann, K.K. and Herrler, G. (2003) Virokinin, a bioactive peptide of the tachykinin family is released from fusion of protein of bovine respiratory syncytial virus. *Journal of Biological Chemistry* 278, 46,854–46,861.

11 Avian Metapneumoviruses

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11.1 Introduction

Avian metapneumovirus (aMPV) is a non-segmented, negative-strand RNA-enveloped virus that is the primary causal agent of turkey rhinotracheitis (TRT), also known as avian rhinotracheitis (ART). The virus was first isolated from turkeys in South Africa during the late 1970s (Buys and du Preez, 1980; Buys *et al.*, 1989a, 1989b). Thereafter, outbreaks were reported in many European, South American and Asian countries and recently also in the USA (Andral *et al.*, 1985; Alexander *et al.*, 1986; Weisman *et al.*, 1988; Senne *et al.*, 1997; Cook *et al.*, 1999; Seal, 2000). Although vaccines have been developed, outbreaks can also be traced to environmental spread of a vaccine-derived virus (Lupini *et al.*, 2011) and the disease is exacerbated by bacterial infections (Cook *et al.*, 1991). aMPV also causes swollen head syndrome in chickens (McDougall and Cook, 1986; Wilding *et al.*, 1986; Wyeth *et al.*, 1987; Cook *et al.*, 1988; Buys *et al.*, 1989b) and outbreaks among chickens have been reported in various parts of the world (Otsuki *et al.*, 1996). In addition, aMPV can infect ducks (Toquin *et al.*, 1999), pheasants (Gough *et al.*, 1988; Welchman *et al.*, 2002), guinea fowl (Picault *et al.*, 1987) or ostriches (Cadman *et al.*, 1994). aMPV RNA or infectious particles have also been isolated from asymptomatic free-ranging water birds, sparrows and starlings in the USA (Shin *et al.*, 2000a; Bennett *et al.*, 2002). The data on the susceptibility of pigeons to aMPV infection is controversial (Felippe *et al.*, 2011; Gharaibeh and Shamoun, 2012).

aMPV causes an acute highly contagious infection of the upper respiratory tract in turkeys that was originally referred to as turkey rhinotracheitis, hence the original name turkey rhinotracheitis virus (TRTV). Disease appeared in the UK during the mid-1980s and spread rapidly, affecting turkey flocks of all ages (Anon, 1985; Naylor and Jones, 1993). The early aMPV isolates from Europe included UK/3B/85 and CVL14/1 strains from the UK (Cavanagh and Barrett, 1988; Collins and Gough, 1988; McDougall and Cook, 1986; Yu *et al.*, 1991), 1556 strain from France, 872S strain from Spain and the 2119 strain from Italy (Collins *et al.*, 1993; Li *et al.*, 1996). All these isolates had high nucleotide sequence and antigenic similarities (Juhasz and Easton, 1994; Li *et al.*, 1996; Randhawa *et al.*, 1996). Nucleotide and amino acid sequence comparisons of the highly variable attachment G gene led to classification of the European viruses as two distinct subgroups, A (aMPV/A) and B (aMPV/B), consistent with the nomenclature adopted for mammalian

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pneumoviruses (Collins *et al.*, 1993; Naylor *et al.*, 1997). It was also demonstrated that these two subgroups had varied patterns of neutralization by monoclonal antibodies recognizing the surface attachment glycoprotein G (Collins *et al.*, 1993; Cook *et al.*, 1993). Contrary to initial reports indicating that French and UK isolates belonged to subtype A, detailed sequence analyses showed that only the UK isolates belonged to subtype A, whereas other continental European strains, including French isolates formed subgroup B (Collins *et al.*, 1993; Juhasz and Easton, 1994; Naylor *et al.*, 1997). Subsequently subgroup B strains were also isolated in the UK (Naylor *et al.*, 1997; Bäyon-Auboyer *et al.*, 1999). Two aMPV strains isolated in France during 1985 lacked antigenic similarity to the A or B strains (Toquin *et al.*, 2000) and had low nucleotide or amino acid sequence identity with aMPV/A, aMPV/B and aMPV/C viruses, hence were classified into a fourth subgroup D (aMPV/D; Bäyon-Auboyer *et al.*, 2000). However, this subgroup has not been reported again since that time.

The USA was considered free of aMPV until the first outbreak occurred among commercial turkeys in the state of Colorado during 1996 (Senne *et al.*, 1997; Cook *et al.*, 1999; Seal, 2000) and prior to that time no serological reactivity was detected among North American commercial poultry (Heckert and Myers, 1993). The outbreak in Colorado, a state with a small commercial turkey population, lasted for only 10 months before eradication, primarily by slaughter and stringent biosecurity measures (Edson, 1997; Goyal *et al.*, 2000; Jirjis *et al.*, 2000). Comparison of the nucleotide and predicted amino acid sequences of the fusion (F) and matrix protein (M) genes from the Colorado aMPV isolate with European isolates indicated that the US viruses had significantly different genetic identity resulting in tentative classification of US viruses as subgroup C (Seal, 2000; Alvarez *et al.*, 2003). At approximately the same time, aMPV outbreaks were reported in the north central US state of Minnesota. During subsequent years (1997–2002), aMPV disease has emerged as a major economic problem for turkey farmers in the state of Minnesota, which is one of the largest turkey-producing states in the USA (Chiang *et al.*, 2000; Goyal *et al.*, 2000; Panigrahy *et al.*, 2000). So far, two distinct sublineages of aMPV/C have been identified in the USA (Padhi and Poss, 2009). Recently, aMPV/C of a different genetic lineage to the US isolates was also detected in Muscovy ducks in France (Toquin *et al.*, 1999) and in Korean pheasants (Lee *et al.*, 2007). This lineage of subtype C aMPV was also later detected in Europe among mallards, greylag geese and common gulls (van Boheemen *et al.*, 2012). The wide geographic distribution and detection of aMPV/C in a variety of wild bird species suggest a fast distribution of this subtype and a potential threat to the poultry industry worldwide (Padhi and Poss, 2009).

11.2 Molecular Biology of aMPV Isolates

The aMPV genome consists of eight viral protein genes arranged in the order 3′-N-P-M-F-M2-SH-G-L-5′ flanked by a leader and trailer at the 3′ and 5′ ends, respectively (Fig. 11.1). The linear genomic RNA molecule is tightly encapsidated by the nucleocapsid (N) protein that associates with the phosphoprotein (P) and large polymerase (L) proteins to form a helical ribonucleoprotein complex, which is the biologically functional unit common to all members of the order *Mononegavirales*. The ribonucleoprotein complex is the template for transcription of mRNAs as well as genome replication (Conzelmann, 1998). aMPV shares several characteristics with other members of the *Pneumovirinae* subfamily (Cavanagh and Barrett, 1988; Yu *et al.*, 1991), including possession of small hydrophobic (SH) and second matrix (M2) genes (Ling *et al.*, 1992; Yu *et al.*, 1992a, 1992b) and structure of the F protein (Naylor *et al.*, 1998). The M2 gene of aMPV-C contains two overlapping open reading frames (ORFs), encoding two putative proteins, M2-1 and M2-2, which are

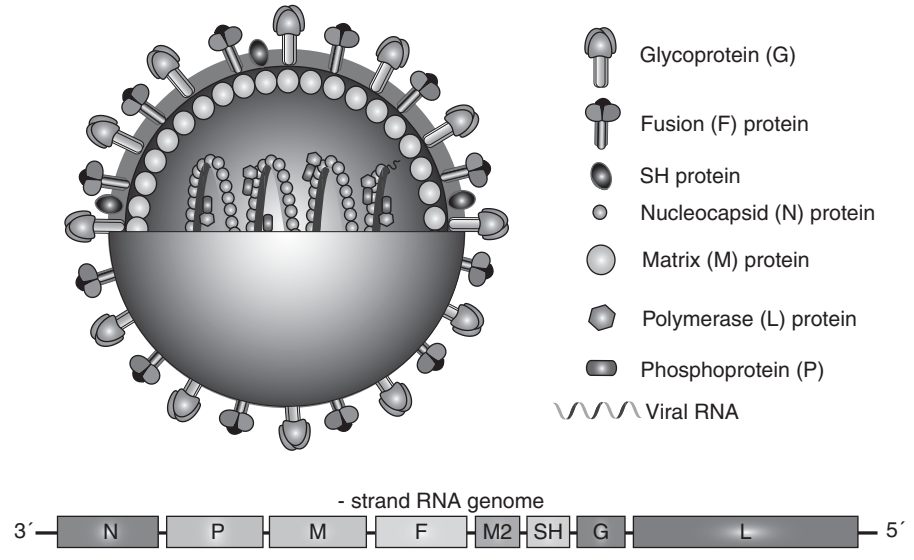

Fig. 11.1. Schematic structure and genomic organization of avian metapneumoviruses.

believed to be involved in viral RNA transcription or replication (Yu *et al.*, 2011). However, aMPV differs from members of the *Pneumovirus* genus, including human and bovine respiratory syncytial virus (hRSV and bRSV), in that it lacks two nonstructural proteins located upstream of the N gene (Randhawa *et al.*, 1997; Marriot *et al.*, 2001). In addition, the SH and G genes of pneumoviruses are located upstream of the F gene, whereas they are located downstream of F gene for aMPV (Ling *et al.*, 1992). As a result of these observations, aMPV was classified into a new genus called *Metapneumovirus* (Pringle, 1999).

European viruses have 97% to 99% nucleotide and amino acid predicted sequence identity within a subtype, whereas strains across subtypes have 56% to 61.2% nucleotide sequence and 33.2% to 38% predicted amino acid sequence identities within the highly variable attachment glycoprotein (G) gene (Juhasz and Easton, 1994). More detailed analyses of nucleotide sequence of N, P, M, fusion glycoprotein (F) and M2 protein genes revealed that these genes could also be used to classify aMPV strains into the four subtypes (Seal, 1998; Bäyon-Auboyer *et al.*, 2000; Shin *et al.*, 2002; Jacobs *et al.*, 2003). For example, US viruses classified as subtype C (aMPV/C) had over 90% nucleotide sequence identity within the five genes, whereas comparison with subtypes A and B revealed between 40% and 70% nucleotide sequence identity (Shin *et al.*, 2002). Phylogenetic analyses of subtypes A, B and C strains demonstrated that A and B viruses were more closely related to each other than either subtype A or B were to C viruses (Seal, 1998; Shin *et al.*, 2002).

Typical for paramyxoviruses, the upstream five genes (N, P, M, F and M2) of aMPV/C were easily sequenced at the nucleotide level due to the abundance of mRNA transcripts, whereas SH, G and L genes were more difficult to obtain due to the paucity of RNA. aMPV was the only recognized member of the *Metapneumovirus* genus, until isolation of a human metapneumovirus (hMPV) in the Netherlands during 2001 (van den Hoogen *et al.*, 2001). Subsequently, hMPV strains were isolated in many countries throughout the world (reviewed by Feuillet *et al.*, 2012). Analysis of the genomic sequences from hMPV demonstrated a close resemblance to aMPV subtype C rather than to aMPV/A, B or D based on nucleotide sequence and phylogenetic comparisons (van den Hoogen *et al.*, 2002;

Toquin *et al.*, 2003). The nucleotide sequence similarity between aMPV and hMPV utilizing N, P, M, F, M2 and L ranged from 56% to 88% (van den Hoogen *et al.*, 2002). The presence of two aMPV N gene-encoded polypeptides was detected in aMPV/C/US/Co- and aMPV/A/UK/3b-infected Vero cells (Alvarez and Seal, 2005) and also in hMPV-infected cells (Tedcastle *et al.*, 2012).

11.3 Pathobiology of aMPV

Natural aMPV-infection, as indicated by experimental inoculations of susceptible hosts, is via the upper respiratory tract (Jones *et al.*, 1986). As with other related viruses of the *Pneumovirinae* subfamily, the aMPV G-protein is considered to mediate virus attachment to host cells (Collins and Crowe, 2007), which are mainly ciliated epithelia cells (Fig. 11.2), but macrophages are also hypothesized to be permissive for aMPV replication (Jirjis *et al.*, 2002a). However, potential receptors have not yet been identified (Winter *et al.*, 2008). The F-protein mediates fusion of the viral envelope with the cell membrane, and contributes to host tropism, leading to fusion of infected and non-infected neighbouring cells with typical syncytia formation (Tanaka *et al.*, 1996; de Graaf *et al.*, 2009).

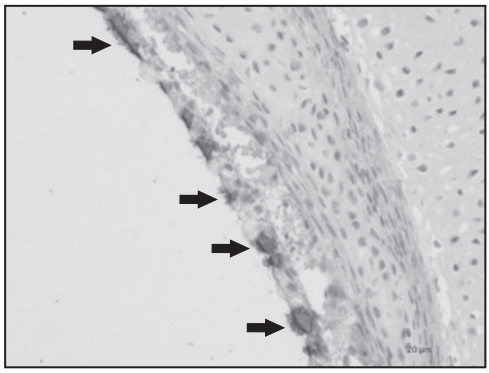

Fig. 11.2. Detection of avian metapneumovirus (aMPV) by immunohistochemistry. Arrows indicate aMPV-infected ciliated cells of tracheal organ cultures at 96 hours post-infection.

Host proteases are necessary for activation of the F-protein by cleavage, but sequence analysis of vaccine and field strains suggests that the F-protein cleavage site may not be an important feature of viral virulence (Sugiyama *et al.*, 2010).

European aMPV strains cause deciliation of the trachea among infected turkeys at approximately 96 hours post-infection, with the virus inducing watery to mucoid exudates in the turbinates and excess mucus in the trachea from 1–9 days post-infection (Jones *et al.*, 1986, 1987). By direct immunofluorescence staining, virus is detected in the trachea between days 1 and 7 post-infection and in the turbinates between days 2 and 5 post-infection; however, no virus was demonstrated in the lungs or air sacs. aMPV replication in ciliated epithelial cells of the female chicken and turkey reproductive tracts has also been demonstrated under field and experimental conditions (Jones *et al.*, 1988; Cook *et al.*, 2000). Viral antigens were detected by immunofluorescence in the epithelium of the uterus on day 7 post-infection, and all other regions of the oviduct on day 9 post-infection (Jones *et al.*, 1988). These infections may lead to significant drops in egg production, egg peritonitis, misshapen eggs accompanied by ovary or oviduct regression (Hess *et al.*, 2004; Sugiyama *et al.*, 2006). Field outbreaks are exacerbated due to secondary bacterial pathogens inducing air sacculitis, pericarditis, pneumonia and perihepatitis (Cook, 2000). Infections with mycoplasma (Naylor *et al.*, 1992) or *Escherichia coli*, *Bordetella avium*, *Ornithobacterium rhinotracheale* or a mixture of all three can increase loss of cilia on the epithelial surface of the upper respiratory tract, allowing deeper penetration of the virus into the respiratory tract and exacerbating infection by aMPV (Jirjis *et al.*, 2004). Microscopic examination of the turbinates from infected turkeys usually reveals increased glandular activity with loss of cilia, congestion and mononuclear infiltration of the submucosa (Majo *et al.*, 1995, 1996). The US subtype C aMPV produces multifocal loss of cilia in the nasal turbinates of infected turkeys and immunohistochemistry revealed intense

staining of aMPV antigen in turbinates and tracheae of birds infected with wild-type isolates (Jirjis *et al.*, 2002a; Velayudhan *et al.*, 2008). Gross lesions among chickens include a purulent oedema in the subcutaneous tissues of the head, neck and wattles, with varying degrees of swelling in the infraorbital sinuses (Hafez, 1992; Tanaka *et al.*, 1995). Clinical signs such as depression, coughing, nasal exudates and frothy eyes appeared at 4 days post-infection in chickens, followed by swelling of periorbital sinuses at 5 days post-infection and serological conversion occurring within 10 days post-infection (Catelli *et al.*, 1998; Aung *et al.*, 2008). Interestingly, nasal turbinates of hMPV-infected turkeys showed inflammatory changes and mucus accumulation along with positive immunohistochemistry, demonstrating that the human virus closely related to aMPV/C will infect and replicate in turkeys (Velayudhan *et al.*, 2006).

Virus neutralizing antibodies following aMPV infection can be detected locally in tracheal washes and systemically in serum within 5–7 days post-infection, independent of the strain, but titres decrease quickly again on local respiratory surfaces, suggesting only a short duration of local protective immunity (Liman and Rautenschlein, 2007; Rautenschlein *et al.*, 2011). Virus neutralizing antibody titres peak at 7 days post-infection and then antibody levels decline, with peak serum ELISA antibody production varying among infected groups, ranging from 14 and 28 days post-infection. Virulent strains may markedly decrease the proliferative response of spleen cells to concanavalin A (Chary *et al.*, 2002). aMPV strains induce an increase in the percentage of $CD4^+$ T cell populations in the spleen and Harderian gland (HG) at days 7 or 14 post-infection in turkeys and of $CD4^+$ and $CD8^+$ cells in the HG in chickens in the early phase (Rautenschlein *et al.*, 2011; Liman and Rautenschlein, 2007). Investigations with chemically bursectomized turkeys indicate that cell-mediated immunity in turkeys is important for protection against aMPV infection (Jones *et al.*, 1992). Furthermore, T-cell-deficient turkeys partially depleted of functional $CD4^+$ and $CD8^+$ T-lymphocytes by cyclosporin A (CsA) treatment showed a delay in viral clearance, recovery from aMPV-induced clinical signs and histopathological lesions compared to T-cell-intact birds (Rubbenstroth *et al.*, 2010). Differences in systemic and local T cell and possibly natural killer cell activity in the HG between turkeys and chickens may explain the differences in aMPV-pathogenesis between chickens and turkeys (Rautenschlein *et al.*, 2011).

11.4 Diagnostics and Prevention of aMPV Disease

There are a variety of diagnostic strategies utilized for aMPV detection in clinical samples (Fig. 11.3; Cook and Cavanagh, 2002). The time period for successful virus detection after infection is short and for broilers experimental studies indicate that virus detection may not be successful beyond the peak of clinical signs, which is expected around 6 days post-infection (Aung *et al.*, 2008). Upper respiratory tract tissue, tracheal, sinus and choanal swabs may be most appropriate to detect aMPV (Van de Zande *et al.*, 1999; Pedersen *et al.*, 2001). Isolation of this slowly replicating virus was first successful utilizing tracheal organ cultures (Buys and du Preez, 1980; Buys *et al.*, 1989a); however, this technique was not useful for detecting US aMPV/C replication since it does not induce ciliostasis (Cook *et al.*, 1999). Embryonating turkey or chicken eggs can be used to isolate the virus, but serial passage is usually required before embryo lethality can be detected to confirm isolation (Buys and du Preez, 1980; Senne *et al.*, 1997). Chicken embryo fibroblasts and Vero cells in culture can be utilized to successfully propagate aMPV, but blind passage may be necessary prior to observing cytopathic effect for confirmation of virus replication (Goyal *et al.*, 2000; Coswig *et al.*, 2010). The virus visualized by electron microscopy is a characteristic *Paramyxovirus* with a pleomorphic, spherical (100–600 nm) or filamentous (to 1000 nm) particle having helical nucleocapsids

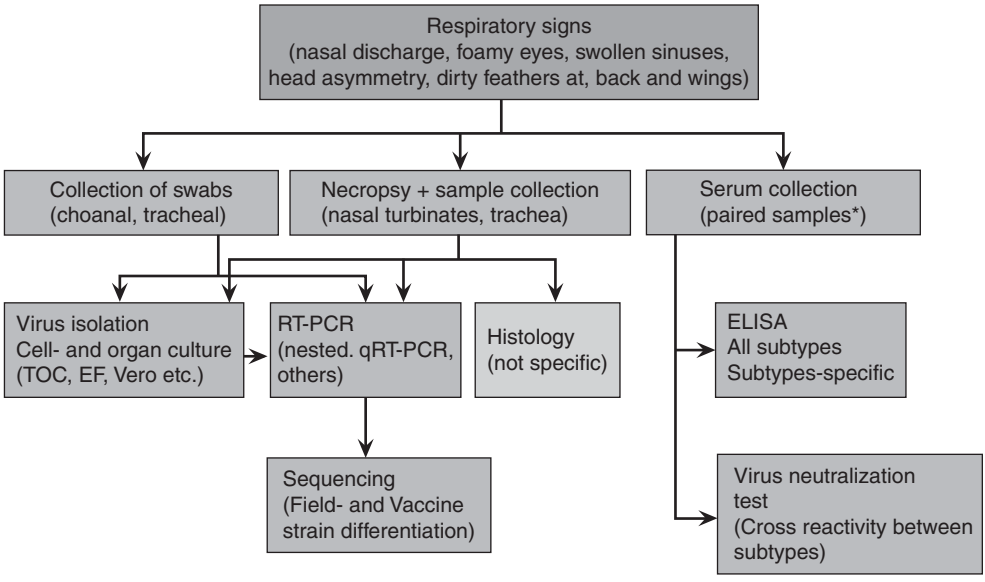

Fig. 11.3. Diagnostic strategy for detection of avian metapneumovirus infections. *Serum samples should be obtained at 2–3-week intervals for detecting an increase in antibody titres.

(14 nm) with glycoprotein projections of 13–14 nm. Immuno-gold labelling of TRT/aMPV in Vero cells was also used to confirm morphology and morphogenesis (O'Loan *et al.*, 1992).

Immunofluorescence of tissues and clinical smears were used to detect European strains as well as aMPV/C isolate of the virus (Usami *et al.*, 1999; Van De Zande *et al.*, 1999; Jirjis *et al.*, 2002b). An ELISA method was developed for detecting antibodies to TRT utilizing antigen from a virus isolated from an outbreak of the disease in Europe (Grant *et al.*, 1987; Chettle and Wyeth, 1988) and this assay was improved upon by utilizing a streptavidin-biotin detection protocol (O'Loan *et al.*, 1989). The choice of antigen may have a negative effect on the ability to detect viral antibodies in turkey sera (Eterradossi *et al.*, 1995). Consequently ELISAs based on recombinant M (Gulati *et al.*, 2000) and N (Gulati *et al.*, 2001a) proteins of aMPV/C were developed. A nucleoprotein (N)-peptide ELISA was created capable of detecting antibodies to all three aMPV types with a reliable antigen (Alvarez *et al.*, 2004b) and polyclonal or monoclonal antibodies reactive to a conserved region of the aMPV/C N cross-reacted with the hMPV N protein, but not with RSV N protein by ELISA, Western blot and immunohistochemical assays (Alvarez *et al.*, 2004a). This cross-reactive association between aMPV and hMPV was also demonstrated utilizing a reverse-genetics system (Govindarajan *et al.*, 2006). Currently a variety of ELISA systems are commercially available to detect antibodies against subtypes A, B and C that can be used to detect field outbreaks as well as vaccine reactions. When completing serology, investigators are reminded that antibody levels may be low or undetectable after only one or two applications of live vaccine (Liman and Rautenschlein, 2007; Ganapathy *et al.*, 2010).

Reverse-transcription, polymerase chain reaction (RT-PCR) was first utilized to detect the F-protein gene of European aMPV subtypes (Jing *et al.*, 1993). Also N- and G-based RT-PCR were specific and sensitive tools for rapid diagnosis and typing of aMPV in field samples (Bäyon-Auboyer *et al.*, 1999). Following the outbreak of TRT in the USA,

several RT-PCR assays were developed to detect aMPV/C in clinical samples (Pedersen *et al.*, 2000; Shin *et al.*, 2000b; Dar *et al.*, 2001). A real-time reverse transcription PCR (RRT-PCR) was determined useful for rapid and sensitive detection, identification and quantitation of the four aMPV subgroups (Guionie *et al.*, 2007) and single round paramyxovirus-wide RT-PCR assay is reportedly capable of detecting aMPV along with other members of the viral family (van Boheemen *et al.*, 2012). Due to the wide spread of aMPV, it has become increasingly important to also differentiate between vaccine and field virus infected flocks by sequencing approaches or restriction enzyme digestion of RT-PCR products (Sugiyama *et al.*, 2010; Listorti *et al.*, 2012).

Attenuated live-virus vaccines were developed by passing European strains in embryonated chicken eggs or tracheal organ cultures (Cook and Ellis, 1990; Williams *et al.*, 1991) that provide good cross-protection between subtypes A and B (Cook *et al.*, 1995). Multiple vaccinations may be necessary to induce sufficient protection throughout the whole growing period of especially male turkeys (Rautenschlein and Günther, 2010). Booster vaccinations may be necessary to be able to detect a humoral immune response by the commercially available ELISA systems, but serum antibodies are not considered to be the primary mode of protection (Rubbenstroth and Rautenschlein, 2009). Possible interactions of aMPV live vaccines with other replicating respiratory pathogens and their vaccine strains may also need to be considered during production (Ganapathy *et al.*, 2007; Lemiere *et al.*, 2012). A combination of priming with an attenuated live-virus followed by injection of inactivated vaccine provided good protection against both respiratory infection and reductions in egg production (Cook *et al.*, 1996).

The F protein of aMPV/C, expressed from a DNA plasmid, was recognized by antiserum to both A and B subgroup aMPVs, but no significant clinical protection was detected following homologous challenge of poults (Tarpey *et al.*, 2001). Live attenuated vaccines for aMPV/C were developed (Gulati *et al.*, 2001b) and a mutant goose variant of aMPV with the largest G gene of any pneumovirus or metapneumovirus was reported as a safe and effective vaccine against aMPV infection outbreaks in commercial turkeys (Bennett *et al.*, 2005). Chicks could be protected against disease earlier in the field if vaccinated by the *in ovo* route versus vaccination at a day post-hatch (Tarpey and Huggins, 2007). Understanding the basis of live-virus attenuation is important because the efficacy of a putative protective vaccine strain can be affected by mutations altering the balance of G protein expression (Naylor *et al.*, 2007).

A recombinant fowlpox virus was produced that expressed the F protein of aMPV/TRTV that induced antibodies, which were detectable both by an ELISA and a virus neutralization test. Turkeys vaccinated with the F recombinant showed milder clinical signs and 1000-fold less challenge virus was recovered from the nose and trachea compared with turkeys that had been vaccinated with control fowlpox virus (Qingzhong *et al.*, 1994). Apparently modification of the gene that encodes the M2 protein can lead to an improvement in production of vaccines by reverse genetics systems (Clubbe and Naylor, 2011; Yu *et al.*, 2011). Utilizing a reverse genetics system, an NDV-based, LaSota strain recombinant vaccine virus expressing the glycoprotein (G) of aMPV-C was generated as a bivalent vaccine that provided partial protection against pathogenic aMPV-C challenge and complete protection against velogenic NDV challenge (Hu *et al.*, 2011). Although a formalin-inactivated vaccine does not provide protection (Kapczynski *et al.*, 2008), a virosome preparation (Kapczynski, 2004) and use of an oculonasally delivered poly(D,L-lactic-co-glycolic acid) microparticle (PLGA-MP)-based system with an aMPV recombinant F protein resulted in reduced clinical signs of disease (Liman *et al.*, 2007). Viral replication can be inhibited by interfering RNA (Munir *et al.*, 2006; Ferreira *et al.*, 2007), which could potentially be a future strategy for control of the virus.

11.5 Concluding Remarks

Although known for over 30 years and the application of different hygiene and vaccination strategies, aMPV still appears to be a problem in the field. Control measures are not always successful due to the fast horizontal spread of aMPV and its often unexpected long persistence (Lupini *et al.*, 2011). Vaccine failures, virus evolution in response to vaccine pressure and the possible risk of reversion of vaccines to more virulent strains may interfere with conventional vaccination strategies (Catelli *et al.*, 2006, 2010; Cecchinato *et al.*, 2010). Consequently, the ongoing progress in the development of a new generation of vaccines may provide new possibilities to better control aMPV in the field in future (Liman *et al.*, 2007; Clubbe and Naylor, 2011; Hu *et al.*, 2011).

References

Alexander, D.J., Gough, R.E., Wyeth, P.J., Lister, S.A. and Chettle, N.J. (1986) Viruses associated with turkey rhinotracheitis in Great Britain. *Veterinary Record* 118, 217–218.

Alvarez, R. and Seal, B.S. (2005) Identification of a truncated nucleoprotein in avian metapneumovirus-infected cells encoded by a second AUG, in-frame to the full-length gene. *Virology Journal* 2, 31.

Alvarez, R., Lwamba, H.M., Kapczynski, D.R., Njenga, M.K. and Seal, B.S. (2003) Nucleotide and predicted amino acid sequence-based analysis of the avian metapneumovirus type C cell attachment glycoprotein gene: phylogenetic analysis and molecular epidemiology of US pneumoviruses. *Journal of Clinical Microbiology* 41, 1730–1735.

Alvarez, R., Jones, L.P., Seal, B.S., Kapczynski, D.R. and Tripp, R.A. (2004a) Serological cross-reactivity of members of the metapneumovirus genus. *Virus Research* 105, 67–73.

Alvarez, R., Njenga, M.K., Scott, M. and Seal, B.S. (2004b) Development of a nucleoprotein-based enzyme-linked immunosorbent assay using a synthetic peptide antigen for detection of avian metapneumovirus antibodies in turkey sera. *Clinical and Diagnostic Laboratory Immunology* 11, 245–249.

Andral, B., Louzis, C., Edlinger, E., Newman, J.A., Toquin, D. and Bennejean, G. (1985) Respiratory disease (rhinotracheitis) in turkeys in Brittany, France, 1981–1982. II. Laboratory findings. *Avian Diseases* 29, 35–42.

Anon (1985) Rhinotracheitis of unknown etiology in England and Wales (preliminary report from the British Veterinary Poultry Association). *Veterinary Record* 117, 653–654.

Aung, Y.H., Liman, M., Neumann, U. and Rautenschlein, S. (2008) Reproducibility of swollen sinuses in broilers by experimental infection with avian metapneumovirus subtypes A and B of turkey origin and their comparative pathogenesis. *Avian Pathology* 37, 65–74.

Bäyon-Auboyer, M.H., Jestin, V., Toquin, D., Cherbonnel, M. and Eterradossi, N. (1999) Comparison of F-, G- and N-based RT-PCR protocols with conventional virological procedures for the detection and typing of turkey rhinotracheitis virus. *Archives of Virology* 144, 1091–1109.

Bäyon-Auboyer, M.H., Arnauld, C., Toquin, D. and Eterradossi, N. (2000) Nucleotide sequences of the F, L, and G protein genes of two non-A/non-B avian pneumoviruses (AMPV) reveal a novel AMPV subtype. *Journal of General Virology* 81, 2723–2733.

Bennett, R.S., McComb, B., Shin, H.-J., Njenga, M.K., Nagaraja, K.V. and Halvorson, D.A. (2002) Detection of avian pneumovirus in wild Canadian geese (*Branucleotidea canadensis*) and Blue-Winged teal (*Anas discors*). *Avian Diseases* 46, 1025–1029.

Bennett, R.S., LaRue, R., Shaw, D., Yu, Q., Nagaraja, K.V., Halvorson, D.A. and Njenga, M.K. (2005) A wild goose metapneumovirus containing a large attachment glycoprotein is avirulent but immunoprotective in domestic turkeys. *Journal of Virology* 79, 14,834–14,842.

Buys, S.B. and du Preez, J.H. (1980) A preliminary report on the isolation of a virus causing sinusitis in turkeys in South Africa and attempts to attenuate the virus. *Turkeys* 28, 36–46.

Buys, S.B., du Preez, J.H. and Els, H.J. (1989a) The isolation and attenuation of a virus causing rhinotracheitis in turkeys in South Africa. *Onderstepoort Journal of Veterinary Research* 56, 87–98.

Buys, S.B., du Preez, J.H. and Els, H.J. (1989b) Swollen head syndrome in chickens: a preliminary report on the isolation of a possible aetiological agent. *Journal of South African Veterinary Association* 60, 221–222.

Cadman, H.F., Kelly, P.J., Zhou, R., Davelaar, F. and Mason, P.R. (1994) A serosurvey using enzyme-linked immunosorbent assay for antibodies against poultry pathogens in Ostriches (*Struthio camelus*) from Zimbabwe. *Avian Diseases* 38, 621–625.

Catelli, E., Cook, J.K.A., Chesher, J., Orbell, S.J., Woods, M.A., Baxendale, W. and Huggins, M.B. (1998) The use of virus isolation, histopathology and immunoperoxidase techniques to study the dissemination of a chicken isolate of avian pneumovirus in chickens. *Avian Pathology* 27, 632–640.

Catelli, E., Cecchinato, M., Savage, C.E., Jones, R.C. and Naylor, C.J. (2006) Demonstration of loss of attenuation and extended field persistence of a live avian metapneumovirus vaccine. *Vaccine* 24, 6476–6482.

Catelli, E., Lupini, C., Cecchinato, M., Ricchizzi, E., Brown, P. and Naylor, C.J. (2010) Field avian metapneumovirus evolution avoiding vaccine induced immunity. *Vaccine* 28, 916–921.

Cavanagh, D. and Barrett, T. (1988) Pneumovirus-like characteristics of the mRNA and proteins of turkey rhinotracheitis virus. *Virus Research* 11, 241–256.

Cecchinato, M., Catelli, E., Lupini, C., Ricchizzi, E., Clubbe, J., Battilani, M. and Naylor, C.J. (2010) Avian metapneumovirus (AMPV) attachment protein involvement in probable virus evolution concurrent with mass live vaccine introduction. *Veterinary Microbiology* 146, 24–34.

Chary, P., Rautenschlein, S., Njenga, M.K. and Sharma, J.M. (2002) Pathogenic and immunosuppressive effects of avian pneumovirus in turkeys. *Avian Diseases* 46, 153–161.

Chettle, N.J. and Wyeth, P.J. (1988) Turkey rhinotracheitis: detection of antibodies using an ELISA test. *British Veterinary Journal* 144, 282–287.

Chiang, S.-J., Dar, A.M., Goyal, S.M., Sheikh, M.A., Pedersen, J.C., Panigrahy, B., Senne, D., Halvorson, D.A., Nagaraja, K.V. and Kapur, V. (2000) A modified enzyme-linked immunosorbent assay for the detection of avian pneumovirus antibodies. *Journal of Veterinary Diagnostic Investigation* 12, 381–384.

Clubbe, J. and Naylor, C.J. (2011) Avian metapneumovirus M2:2 protein inhibits replication in Vero cells: modification facilitates live vaccine development. *Vaccine* 29, 9493–9498.

Collins, A.P. and Crowe, J.E.J. (2007) Respiratory syncytial virus and metapneumovirus. In: Fields, B.N., Knipe, D.M. and Howley, P.M. (eds) *Fields Virology*, 5th edn. Lippincott Williams & Wilkins, Philadelphia, Pennsylvania, pp. 1601–1646.

Collins, M.S. and Gough, R.E. (1988) Characterization of a virus associated with rhinotracheitis. *Journal of General Virology* 69, 909–916.

Collins, M.S., Gough, R.E. and Alexander, D.J. (1993) Antigenic differentiation of avian pneumovirus isolates using polyclonal antisera and mouse monoclonal antibodies. *Avian Pathology* 22, 469–479.

Conzelmann, K.K. (1998) Nonsegmented negative-strand RNA viruses: genetics and manipulation of viral genomes. *Annual Reviews of Genetics* 32, 123–162.

Cook, J.K.A. (2000) Avian rhinotracheitis. *OIE Revue Scientifique et Technique* 19, 602–613.

Cook, J.K.A. and Cavanagh, D. (2002) Detection and differentiation of avian pneumoviruses (metapneumoviruses). *Avian Pathology* 31, 117–132.

Cook, J.K.A. and Ellis, M.M. (1990) Attenuation of turkey rhinotracheitis virus by alternative passage in embryonated chicken eggs and tracheal organ cultures. *Avian Pathology* 19, 181–185.

Cook, J.K.A., Dolby, C.A., Southee, D.J. and Mockett, A.P.A. (1988) Demonstration of antibodies to turkey rhinotracheitis virus in serum from commercially reared flocks of chickens. *Avian Pathology* 17, 403–411.

Cook, J.K., Ellis, M.M. and Huggins, M.B. (1991) The pathogenesis of turkey rhinotracheitis virus in turkey poults inoculated with the virus alone or together with two strains of bacteria. *Avian Pathology* 20, 155–166.

Cook, J.K.A., Ellis, M.M., Ling, L. and Cavanagh, D. (1993) Antigenic differentiation of strains of turkey rhinotracheitis virus using monoclonal antibodies. *Avian Pathology* 22, 257–273.

Cook, J.K.A., Huggins, M.B., Woods, M.A., Orbell, S.J. and Mockett, A.P.A. (1995) Protection provided by a commercially available vaccine against different strains of turkey rhinotracheitis virus. *Veterinary Record* 136, 392–393.

Cook, J.K.A., Orthel, F., Orbell, S., Woods, M.A. and Huggins, M.B. (1996) An experimental turkey rhinotracheitis (TRT) infection in breeding turkeys and the prevention of its clinical effects using live-attenuated and inactivated TRT vaccines. *Avian Pathology* 25, 231–243.

Cook, J.K.A., Huggins, M.B., Orbell, S.J. and Senne, D.A. (1999) Preliminary antigenic characterization of an avian pneumovirus isolated from commercial turkeys in Colorado, USA *Avian Pathology* 28, 607–617.

Cook, J.K., Chesher, J., Orthel, F., Woods, M.A., Orbell, S.J., Baxendale, W. and Huggins, M.B. (2000) Avian pneumovirus infection of laying hens: experimental studies. *Avian Pathology* 29, 545–556.

Coswig, L.T., Santos, M.B.D., Hafez, H.M., Ferreira, H.L. and Arns, C.W. (2010) Propagation of avian metapneumovirus subtypes A and B using chicken embryo related and other cell systems. *Journal of Virological Methods* 167, 1–4.

Dar, A.M., Tune, K., Munir, S., Panigrahy, B., Goyal, S.M. and Kapur, V. (2001) PCR-based detection of an emerging avian pneumovirus in US turkey flocks. *Journal of Veterinary Diagnostic Investigation* 13, 201–205.

de Graaf, M., Schrauwen, E.J., Herfst, S., van Amerongen, G., Osterhaus, A.D. and Fouchier, R.A. (2009) Fusion protein is the main determinant of metapneumovirus host tropism. *Journal of General Virology* 90, 1408–1416.

Edson, R.K. (1997) Experience with avian pneumovirus. *Proceedings of the 101st Annual meeting of USAHA* Louisville, Kentucky, pp. 471–472.

Eterradossi, N., Toquin, D., Guittet, M. and Bennejean, G. (1995) Evaluation of different turkey rhinotracheitis viruses used as antigens for serological testing following live vaccination and challenge. *Zentralblatt für Veterinärmedizin, Reihe B* 42, 175–186.

Felippe, P.A., Silva, L.H., Santos, M.B., Sakata, S.T. and Arns, C.W. (2011) Detection of and phylogenetic studies with avian metapneumovirus recovered from feral pigeons and wild birds in Brazil. *Avian Pathology* 40, 445–452.

Ferreira, H.L., Spilki, F.R., de Almeida, R.S., Santos, M.M. and Arns, C.W. (2007) Inhibition of avian metapneumovirus (AMPV) replication by RNA interference targeting nucleoprotein gene (N) in cultured cells. *Antiviral Research* 74, 77–81.

Feuillet, F., Lina, B., Rosa-Calatrava, M. and Boivin, G. (2012) Ten years of human metapneumovirus research. *Journal of Clinical Virology* 53, 97–105.

Ganapathy, K., Cox, W.J., Gough, R.E., Cargill, P., Montiel, E. and Jones, R.C. (2007) Protection in specific pathogen free chickens with live avian metapneumovirus and Newcastle disease virus vaccines applied singly or in combination. *Avian Pathology* 36, 313–317.

Ganapathy, K., Bufton, A., Pearson, A., Lemiere, S. and Jones, R.C. (2010) Vaccination of commercial broiler chicks against avian metapneumovirus infection: a comparison of drinking-water, spray and oculo-oral delivery methods. *Vaccine* 28, 3944–3948.

Gharaibeh, S. and Shamoun, M. (2012) Avian metapneumovirus subtype B experimental infection and tissue distribution in chickens, sparrows, and pigeons. *Veterinary Pathology* 49, 704–709.

Gough, R.E., Collins, M.S., Cox, W.J. and Chettle, N.J. (1988) Experimental infection of turkeys, chickens, ducks, geese, guinea fowl, pheasants and pigeons with turkey rhinotracheitis virus. *Veterinary Record* 123, 58–59.

Govindarajan, D., Buchholz, U.J. and Samal, S.K. (2006) Recovery of avian metapneumovirus subgroup C from cDNA: cross-recognition of avian and human metapneumovirus support proteins. *Journal of Virology* 80, 5790–5797.

Goyal, S.M., Chiang, S.-J., Dar, A.M., Nagaraja, K.V., Shaw, D.P., Halvorson, D.A. and Kapur, V. (2000) Isolation of avian pneumovirus from an outbreak of respiratory illness in Minnesota turkeys. *Journal of Veterinary Diagnostic Investigation* 12, 166–168.

Grant, M., Baxter-Jones, C. and Wilding, G.P. (1987) An enzyme-linked immunosorbent assay for the serodiagnosis of turkey rhinotracheitis infection. *Veterinary Record* 120, 279–280.

Guionie, O., Toquin, D., Sellal, E., Bouley, S., Zwingelstein, F., Allee, C., Bougeard, S., Lemiere, S. and Eterradossi, N. (2007) Laboratory evaluation of a quantitative real-time reverse transcription PCR assay for the detection and identification of the four subgroups of avian metapneumovirus. *Journal of Virological Methods* 139, 150–158.

Gulati, B.R., Cameron, K.T., Seal, B.S., Goyal, S.M., Halvorson, D.A. and Njenga, M.K. (2000) Development of a highly sensitive and specific enzyme-linked immunosorbent assay based on recombinant matrix protein for detection of avian pneumovirus antibodies. *Journal of Clinical Microbiology* 38, 4010–4014.

Gulati, B.R., Munir, S., Patnayak, D.P., Goyal, S.M. and Kapur, V. (2001a) Detection of antibodies to US isolates of avian pneumovirus by a recombinant nucleocapsid protein-based sandwich enzyme-linked immunosorbent assay. *Journal of Clinical Microbiology* 39, 2967–2970.

Gulati, B.R., Patnayak, D.P., Sheikh, A.M., Poss, P.E. and Goyal, S.M. (2001b) Protective efficacy of high-passage avian pneumovirus (APV/MN/turkey/1-a/97) in turkeys. *Avian Diseases* 45, 593–597.

Hafez, H.M. (1992) Comparative investigation on different turkey rhinotracheitis (TRT) virus isolates from different countries. *Deutsche Tierarztliche Wochenschrift* 99, 486–488.

Heckert, R.A. and Myers, D.J. (1993) Absence of antibodies to avian pneumovirus in Canadian poultry. *Veterinary Record* 132, 172.

Hess, M., Huggins, M.B., Mudzamiri, R. and Heincz, U. (2004) Avian metapneumovirus excretion in vaccinated and non-vaccinated specified pathogen free laying chickens. *Avian Pathology* 33, 35–40.

Hu, H., Roth, J.P., Estevez, C.N., Zsak, L., Liu, B. and Yu, Q. (2011) Generation and evaluation of a recombinant Newcastle disease virus expressing the glycoprotein (G) of avian metapneumovirus subgroup C as a bivalent vaccine in turkeys. *Vaccine* 29, 8624–8633.

Jacobs, J.A., Njenga, M.K., Alvarez, R., Mawditt, K., Britton, P., Cavanagh, D. and Seal, B.S. (2003) Subtype B avian metapneumovirus resembles subtype A more closely than subtype C or human metapneumovirus with respect to the phosphoprotein, and second matrix and hydrophobic proteins. *Virus Research* 92, 171–178.

Jing, Li, Cook, J.K.A., Brown, T.D.K., Shaw, K. and Cavanagh, D. (1993) Detection of turkey rhinotracheitis virus in turkeys using the polymerase chain reaction. *Avian Pathology* 22, 771–783.

Jirjis, F.F., Noll, S.L., Halvorson, D.A., Nagaraja, K.V., Townsend, E.L., Sheikh, A.M. and Shaw, D.P. (2000) Avian pneumovirus infection in Minnesota turkeys: experimental reproduction of the disease. *Avian Diseases* 44, 222–226.

Jirjis, F.F., Noll, S.L., Halvorson, D.A., Nagaraja, K.V. and Shaw, D.P. (2002a) Pathogenesis of avian pneumovirus infection in turkeys. *Veterinary Pathology* 39, 300–310.

Jirjis, F.F., Noll, S.L., Halvorson, D.A., Nagaraja, K.V., Townsend, E.L., Goyal, S.M. and Shaw, D.P. (2002b) Rapid detection of avian pneumovirus in tissue culture by microindirect immunofluorescence test. *Journal of Veterinary Diagnostic Investigation* 14, 172–175.

Jirjis, F.F., Noll, S.L., Halvorson, D.A., Nagaraja, K.V., Martin, F. and Shaw, D.P. (2004) Effects of bacterial coinfection on the pathogenesis of avian pneumovirus infection in turkeys. *Avian Diseases* 48, 34–49.

Jones, R.C., Baxter-Jones, C., Wilding, G.P. and Kelly, D.F. (1986) Demonstration of a candidate virus for turkey rhinotracheitis in experimentally inoculated turkeys. *Veterinary Record* 119, 599–600.

Jones, R.C., Baxter-Jones, C., Savage, C.E., Kelly, D.F. and Wilding, G.P. (1987) Experimental infection of chickens with a ciliostatic agent isolated from turkeys with rhinotracheitis. *Veterinary Record* 120, 301–302.

Jones, R.C., Williams, R.A., Baxter-Jones, C., Savage, C.E. and Wilding, G.P. (1988) Experimental infection of laying turkeys with rhinotracheitis virus: distribution of virus in the tissues and serological response. *Avian Pathology* 17, 841–850.

Jones, R.C., Naylor, C.J., al-Afaleq, A., Worthington, K.J. and Jones, R. (1992) Effect of cyclophosphamide immunosuppression on the immunity of turkeys to viral rhinotracheitis. *Research in Veterinary Science* 53, 38–41.

Juhasz, K. and Easton, A.J. (1994) Extensive sequence variation in the attachment (G) protein gene of avian pneumovirus: evidence for two distinct subtypes. *Journal of General Virology* 75, 2873–2880.

Kapczynski, D.R. (2004) Development of a virosome vaccine against avian metapneumovirus subtype C for protection in turkeys. *Avian Diseases* 48, 332–343.

Kapczynski, D.R., Perkins, L.L. and Sellers, H.S. (2008) Mucosal vaccination with formalin-inactivated avian metapneumovirus subtype C does not protect turkeys following intranasal challenge. *Avian Diseases* 52, 28–33.

Lee, E., Song, M.S., Shin, J.Y., Lee, Y.M., Kim, C.J., Lee, Y.S., Kim, H. and Choi, Y.K. (2007) Genetic characterization of avian metapneumovirus subtype C isolated from pheasants in a live bird market. *Virus Research* 128, 18–25.

Lemiere, S., Awad, F., Forrester, A., Jones, R. and Ganapathy, K. (2012) Interactions between live avian metapneumovirus, Newcastle disease and infectious bronchitis vaccine viruses in specific pathogen free chickens. *Abstracts of the VIIth International Symposium on Avian Corona- and Pneumoviruses and Complicating Pathogens*, Rauischholzhausen, Germany, 18–21 June 2012, p. 61.

Li, J., Ling, R., Randhawa, J.S., Shaw, K., Davis, P.J., Juhasz, K., Pringle, C.R., Easton, A.J. and Cavanagh, D. (1996) Sequence of the nucleocapsid protein gene of subgroup A and B avian pneumo-viruses. *Virus Research* 41, 185–191.

Liman, M and Rautenschlein, S. (2007) Induction of local and systemic immune reactions following infection of turkeys with avian metapneumovirus (aMPV) subtypes A and B. *Veterinary Immunology and Immunopathology* 115, 273–285.

Liman, M., Peiser, L., Zimmer, G., Propsting, M., Naim, H.Y. and Rautenschlein, S. (2007) A genetically engineered prime-boost vaccination strategy for oculonasal delivery with poly (d,l-lactic-co-glycolic acid) microparticles against infection of turkeys with avian metapneumovirus. *Vaccine* 25, 7914–7926.

Ling, R., Easton, A.J. and Pringle, C.R. (1992) Sequence analysis of the 22K, SH and G genes of turkey rhinotracheitis virus and their intergenic regions reveal a gene order different from that of other pneumoviruses. *Journal of General Virology* 73, 1709–1715.

Listorti, V., Lupini, C., Cacchinato, M., Pesente, P., Rossi, G., Naylor, C.J. and Catelli, E. (2012) Rapid differentiation of vaccine and field strains of avian metapneumovirus of subtype B by restriction enzyme digestion of PCR products. *Abstracts of the VIIth International Symposium on Avian Corona- and Pneumoviruses and Complicating Pathogens*, Rauischholzhausen, Germany, 18–21 June 2012, p. 58.

Lupini, C., Cecchinato, M., Ricchizzi, E., Naylor, C.J. and Catelli, E. (2011) A turkey rhinotracheitis outbreak caused by the environmental spread of a vaccine-derived avian metapneumovirus. *Avian Pathology* 40, 525–530.

Majo, N., Allan, G.M., O'Loan, C.J., Pages, A. and Ramis, A.J. (1995) A sequential histopathologic and immunocytochemical study of chickens, turkey poults, and broiler breeders experimentally infected with turkey rhinotracheitis virus. *Avian Diseases* 39, 887–896.

Majo, N., Marti, M., O'Loan, C.J., Allan, G.M., Pages, A. and Ramis, A. (1996) Ultrastructural study of turkey rhinotracheitis virus infection in turbinates of experimentally infected chickens. *Veterinary Microbiology* 52, 37–48.

Marriot, A.C., Smith, J.M. and Easton, A.J. (2001) Fidelity of leader and trailer sequence usage by the respiratory syncytial virus and avian pneumovirus replication complexes. *Journal of Virology* 75, 6265–6272.

McDougall, J.S. and Cook, J.K. (1986) Turkey rhinotracheitis: preliminary investigations. *Veterinary Record* 118, 206–207.

Munir, S., Kaur, K. and Kapur, V. (2006) Avian metapneumovirus phosphoprotein targeted RNA interference silences the expression of viral proteins and inhibits virus replication. *Antiviral Research* 69, 46–51.

Naylor, C.J. and Jones, R.C. (1993) Turkey rhinotracheitis: a review. *Veterinary Bulletin* 63, 339–349.

Naylor, C.J., Al-Ankari, A.R., Al-Afaleq, A.I., Bradbury, J.M. and Jones, R.C. (1992) Exacerbation of *Mycoplasma gallisepticum* infection in turkeys by rhinotracheitis virus. *Avian Pathology* 21, 295–305.

Naylor, C.J., Shaw, K., Britton, P. and Cavanagh, D. (1997) Appearance of type B avian pneumovirus in Great Britain. *Avian Pathology* 26, 327–338.

Naylor, C.J., Britton, P. and Cavanagh, D. (1998) The ectodomains but not the transmembrane domains of the fusion proteins of subtypes A and B avian pneumovirus are conserved to a similar extent as those of human respiratory syncytial virus. *Journal of General Virology* 79, 1393–1398.

Naylor, C.J., Ling, R., Edworthy, N., Savage, C.E. and Easton, A.J. (2007) Avian metapneumovirus SH gene end and G protein mutations influence the level of protection of live-vaccine candidates. *Journal of General Virology* 88, 1767–1775.

O'Loan, C.J., Allan, G., Baxter-Jones, C. and McNulty, M.S. (1989) An improved ELISA and serum neutralisation test for the detection of turkey rhinotracheitis virus antibodies. *Journal of Virological Methods* 25, 271–282.

O'Loan, C.J., Curran, W.L. and McNulty, M.S. (1992) Immuno-gold labelling of turkey rhinotracheitis virus. *Zentralblatt fur Veterinarmedizin, Reihe B* 39, 459–466.

Otsuki, K., Hirai, N., Mitani, M., Itani, M., Shimohata, T., Kunii, E., Uramoto, K., Kiyotake, M., Kato, H., Ellis, M.M. and Cook, J.K.A. (1996) Demonstration of serum-neutralising antibody to turkey rhinotracheitis virus in serum from chicken flocks in Japan. *Journal of Veterinary Medical Science* 58, 869–874.

Padhi, A. and Poss, M. (2009) Population dynamics and rates of molecular evolution of a recently emerged paramyxovirus, avian metapneumovirus subtype C. *Journal of Virology* 83, 2015–2019.

Panigrahy, B., Senne, D.A., Pedersen, J.C., Gidlewski, T. and Edson, R.K. (2000) Experimental and serologic observations on avian pneumovirus (APV/turkey/Colorado/97) infection in turkeys. *Avian Diseases* 44, 17–22.

Pedersen, J.C., Reynolds, D.L. and Ali, A. (2000) The sensitivity and specificity of a reverse transcription-polymerase chain reaction assay for the avian pneumovirus (Colorado strain). *Avian Diseases* 44, 681–685.

Pedersen, J.C., Senne, D.A., Panigrahy, B. and Reynolds, D.L. (2001) Detection of avian pneumovirus in tissues and swab specimens from infected turkeys. *Avian Diseases* 45, 581–592.

Picault, J.-P., Giraud, P., Guittet, M., Bennejean, G., Lamande, J., Toquin, D. and Gueguen, C. (1987) Isolation of a TRTV-like virus from chickens with swollen head syndrome. *Veterinary Record* 121, 135.

Pringle, C.R. (1999) Virus taxonomy – 1999. The universal system of virus taxonomy, updated to include the new proposals ratified by the International Committee on Taxonomy of Viruses during 1998. *Archives of Virology* 144, 421–429.

Qingzhong, Y., Barrett, T., Brown, T.D.K., Cook, J.K.A., Green, P., Skinner, M.A. and Cavanagh, D. (1994) Protection against turkey rhinotracheitis pneumovirus (TRTV) induced by a fowlpox virus recombinant expressing the TRTV fusion glycoprotein (F). *Vaccine* 12, 569–573.

Randhawa, J.S., Wilson, S.D., Tolley, K.P., Cavanagh, D., Pringle, C.R. and Easton, A.J. (1996) Nucleotide sequence of the gene encoding the viral polymerase of avian pneumovirus. *Journal of General Virology* 77, 3047–3051.

Randhawa, J.S., Marriott, A.C., Pringle, C.R. and Easton, A.J. (1997) Rescue of synthetic minireplicons establishes the absence of NS1 and NS2 genes from avian pneumovirus. *Journal of Virology* 71, 9849–9854.

Rautenschlein, S. and Günther, R. (2010) Immunologische Grundlagen und Impfprophylaxe beim infektiösen Putenschnupfen (TRT). Proceedings 5. Leipziger Tierärztekongress, Band 2, 21–23 January 2010, Leipzig, Germany, pp. 211–215.

Rautenschlein, S., Aung, Y.H. and Haase, C. (2011) Local and systemic immune responses following infection of broiler-type chickens with avian metapneumovirus subtypes A and B. *Veterinary Immunology Immunopathology* 140, 10–22.

Rubbenstroth, D., and Rautenschlein, S. (2009) Investigations on the protective role of passively transferred antibodies against avian metapneumovirus infection in turkeys. *Avian Pathology* 38, 427–436.

Rubbenstroth, D., Dalgaard, T.S., Kothlow, S., Juul-Madsen, H.R. and Rautenschlein, S. (2010) Effects of cyclosporin A induced T-lymphocyte depletion on the course of avian metapneumovirus (aMPV) infection in turkeys. *Developmental and Comparative Immunology* 34, 518–529.

Seal, B.S. (1998) Matrix protein gene nucleotide and predicted amino acid sequence demonstrate that the first US avian pneumovirus isolate is distinct from European strains. *Virus Research* 58, 45–52.

Seal, B.S. (2000) Avian pneumoviruses and emergence of a new type in the United States of America. *Animal Health Research Reviews* 1, 67–72.

Senne, D.A., Edson, R.K., Pederson, J.C. and Panigrahy, B. (1997) Avian pneumovirus update. In: *Proceedings of the American Veterinary Medical Association* 134th Annual Conference, Reno, Nevada, p. 190.

Shin, H.J., Njenga, M.K., McComb, B., Halvorson, D.A. and Nagaraja, K.V. (2000a) Avian pneumovirus RNA from wild and sentinel birds in the US has genetic homology with aMPV isolates from domestic turkeys. *Journal of Clinical Microbiology* 38, 4282–4284.

Shin, H.J., Rajashekara, G., Jirjis, F.F., Shaw, D.P., Goyal, S.M., Halvorson, D.A. and Nagaraja, K.V. (2000b) Specific detection of avian pneumovirus (APV) US isolates by RT-PCR. *Archives of Virology* 145, 1239–1246.

Shin, H.J., Cameron, K.T., Jacobs, J.A., Turpin, E.A., Halvorson, D.A., Goyal, S.M., Nagaraja, K.V., Mahesh, C.K., Lauer, D.C., Seal, B.S. and Njenga, M.K. (2002) Molecular epidemiology of subtype C avian pneumoviruses isolated from the United States and comparison with subtype A and B viruses. *Journal of Clinical Microbiology* 40, 1687–1693.

Sugiyama, M., Koimaru, H., Shiba, M., Ono, E., Nagata, T. and Ito, T. (2006) Drop of egg production in chickens by experimental infection with an avian metapneumovirus strain PLE8T1 derived from swollen head syndrome and the application to evaluate vaccine. *Journal of Veterinary Medical Science* 68, 783–787.

Sugiyama, M., Ito, H., Hata, Y., Ono, E. and Ito, T. (2010) Complete nucleotide sequences of avian metapneumovirus subtype B genome. *Virus Genes* 41, 389–395.

Tanaka, M., Takuma, H., Kokumai, N., Oishi, E., Obi, T., Hiramatsu, K. and Shimizu, Y. (1995) Turkey rhinotracheitis virus isolated from broiler chicken with swollen head syndrome in Japan. *Journal of Veterinary Medical Science* 57, 939–941.

Tanaka, M., Kokumai, N., Obi, T., Higashihara, R., Takuma, H., Hiramatsu, K. and Shimizu, Y. (1996) A serological survey of turkey rhinotracheitis infection in chicken in Japan. *Journal of Veterinary Medical Science* 58, 689–691.

Tarpey, I. and Huggins, M.B. (2007) Onset of immunity following *in ovo* delivery of avian metapneumovirus vaccines. *Veterinary Microbiology* 124, 134–139.

Tarpey, I., Huggins, M.B., Davis, P.J., Shilleto, R., Orbell, S.J. and Cook, J.K.A. (2001) Cloning, expression and immunogenicity of the avian pneumovirus (Colorado isolate) F protein. *Avian Pathology* 30, 471–474.

Tedcastle, A.B., Fenwick, F., Ingram, R.E., King, B.J., Robinson, M.J. and Toms, G.L. (2012) The characterization of monoclonal antibodies to human metapneumovirus and the detection of multiple forms of the virus nucleoprotein and phosphoprotein. *Journal of Medical Virology* 84, 1061–1070.

Toquin, D., Bäyon-Auboyer, M.H., Eterradossi, N. and Jestin, V. (1999) Isolation of a pneumovirus from a Muscovy duck. *Veterinary Record* 145, 680.

Toquin, D., Bäyon-Auboyer, M.H., Senne, D.A. and Eterradossi, N. (2000) Lack of antigenic relationship between French and recent North American non-A/non-B turkey rhinotracheitis viruses. *Avian Diseases* 44, 977–982.

Toquin, D., de Boisseson, C., Beven, V., Senne, D.A. and Eterradossi, N. (2003) Subtype C avian metapneumovirus (MPV) and the recently isolated human MPV exhibit a common organization but have extensive sequence divergence in their putative SH and G genes. *Journal of General Virology* 84, 2169–2178.

Usami, Y., Mase, M., Yamaguchi, O. and Imai, K. (1999) Detection of antibodies to avian pneumovirus by a micro-indirect immunofluorescent antibody test. *Avian Diseases* 43, 384–390.

van Boheemen, S., Bestebroer, T.M., Verhagen, J.H., Osterhaus, A.D., Pas, S.D., Herfst, S. and Fouchier, R.A.M. (2012) A family-wide RT-PCR assay for detection of paramyxoviruses and application to a large-scale surveillance study. *PLoS ONE* 7, e34961.

van den Hoogen, B.G., DeJong, J.C., Groen, J., Kuiken, T., De Groot, R., Fouchier, R.A.M. and Osterhaus, A.D.M.E. (2001) A newly discovered human metapneumovirus isolated from young children with respiratory tract disease. *Nature Medicine* 7, 719–724.

Van De Zande, S., Nauwynck, H., De Jonghe, S. and Pensaert, M. (1999) Comparative pathogenesis of a subtype a with A subtype B avian pneumovirus in turkeys. *Avian Pathology* 28, 239–244.

van den Hoogen, B.G., Bestebroer, T.M., Osterhaus, A.D.M.E. and Foucher, R.A.M. (2002) Analysis of genome sequence of human metapneumovirus. *Virology* 295, 119–132.

Velayudhan, B.T., Nagaraja, K.V., Thachil, A.J., Shaw, D.P., Gray, G.C. and Halvorson, D.A. (2006) Human metapneumovirus in turkey poults. *Emerging Infectious Diseases* 12, 1853–1859.

Velayudhan, B.T., Noll, S.L., Thachil, A.J., Halvorson, D.A., Shaw, D.P., Goyal, S.M. and Nagaraja, K.V. (2008) Comparative pathogenicity of early and recent isolates of avian metapneumovirus subtype C in turkeys. *Canadian Journal of Veterinary Research* 72, 371–375.

Weisman, Y., Strengel, C., Blumenkranz, R. and Segal, Y. (1988) Turkey rhinotracheitis (TRT) in turkey flocks in Israel–virus isolation and serological response. *Proceedings of 37th Western Poultry Disease Conference*, Davis, California, pp. 67–69.

Welchman, D.B., Bradbury, J.M., Cavanagh, D. and Aebischer, N.J. (2002) Infectious agents associated with respiratory disease in pheasants. *Veterinary Record* 150, 658–664.

Wilding, G.P., Baxter-Jones, C. and Grant, M. (1986) Ciliostatic agent found in rhinotracheitis. *Veterinary Record* 118, 735.

Williams, R.A., Savage, C.E., Worthington, K.J. and Jones, R.C. (1991) Further studies on the development of a live attenuated vaccine against turkey rhinotracheitis. *Avian Pathology* 20, 585–596.

Winter, C., Herrler, G. and Neumann, U. (2008) Infection of the tracheal epithelium by infectious bronchitis virus is sialic acid dependent. *Microbes and Infection* 10, 367–373.

Wyeth, P.J., Gough, R.E. and Collins, M.S. (1987) Antibodies to TRT in chicken with swollen head syndrome. *Veterinary Record* 120, 286–287.

Yu, Q., Davis, P.J., Barreth, T., Binns, M.M., Boursnell, M.E.G. and Cavanagh, D. (1991) Deduced amino acid sequence of the fusion glycoprotein of turkey rhinotracheitis virus has greater identity with that of human respiratory syncytial virus, a pneumovirus, than that of paramyxoviruses and morbilliviruses. *Journal of General Virology* 72, 75–81.

Yu, Q., Davis, P.J., Li, J. and Cavanagh, D. (1992a) Cloning and sequencing of the matrix protein (M) gene of turkey rhinotracheitis virus reveal a gene order different from that of respiratory syncytial virus. *Virology* 186, 426–434.

Yu, Q., Davis, P.J., Brown, T.D.K. and Cavanagh, D. (1992b) Sequence and in vitro expression of the M2 gene of turkey rhinotracheitis pneumovirus. *Journal of General Virology* 73, 1355–1363.

Yu, Q., Estevez, C.N., Roth, J.P., Hu, H. and Zsak, L. (2011) Deletion of the M2-2 gene from avian metapneumovirus subgroup C impairs virus replication and immunogenicity in turkeys. *Virus Genes* 42, 339–346.

12 Bovine Ephemeral Fever Virus

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12.1 Introduction

Bovine ephemeral fever virus (BEFV) is an arthropod-borne rhabdovirus, which has been classified as the type species of the genus *Ephemerovirus* (Wunner *et al.*, 1995). This genus includes the type species BEFV and antigenically related species Adelaide River virus (ARV) and Berrimah virus (Tordo *et al.*, 2008). Like other rhabdoviruses, BEFV virions are bullet- or cone-shaped (Murphy *et al.*, 1972), consisting of a ribonucleoprotein complex and an envelope and containing a negative single-stranded RNA genome and five structural proteins (Della-Porta and Brown, 1979; Walker *et al.*, 1991). It occurs in tropical and subtropical regions of Africa, Asia, Australia and the Middle East, causing a disabling febrile infection in cattle and water buffalo (St George, 1990). The other recognized ephemeroviruses are not known to cause disease. BEFV is serologically related to several other rhabdoviruses including ARV, Berrimah virus and Kimberley virus (Calisher *et al.*, 1989) and these have also been grouped in the genus *Ephemerovirus* (Wunner *et al.*, 1995).

12.2 Virus Biology

12.2.1 Genome organization and coding assignment

BEFV consists of a single-stranded, negative-sense RNA genome. The negative-strand rhadovirus RNA contains five to six genes in the order 3′-l-N-P-M-G-G(NS)-α1-α2-β-γ-L-t-5′ (McWilliam *et al.*, 1997). The genes encode five structural proteins, including the large RNA-dependent RNA polymerase (L), polymerase-associated protein (P), envelope glycoprotein (G), nucleoprotein (N) and matrix protein (M) (Walker *et al.*, 1991). Between the G and L genes, BEFV and ARV contain multiple open reading frames (ORFs) encoding a non-structural transmembrane glycoprotein (G_{NS}), a putative viroporin (α1) and several other small proteins (α2 and β in ARV; α2, α3, β and γ in BEFV) of unknown function (Walker *et al.*, 1991; McWilliam *et al.*, 1997). Despite the complex genome organization, sequence comparisons of G, G_{NS}, N and 3′-leader sequences have revealed that BEFV and ARV are most closely related to vesicular stomatitis virus (VSV) and other vesiculoviruses (Walker *et al.*, 1992, 1994; Wang and

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Walker, 1993; Wang *et al.*, 1995). Although there appear to be differences in phosphorylation, sequences of ephemerovirus P and M proteins are also most closely related to vesiculoviruses (Wang *et al.*, 1995; Dhillon, 1996).

Ephemeroviruses share the unusual characteristic of a large genome encoding two transmembrane glycoproteins (G and GNS) that are related in amino acid sequences to each other and to other rhabdovirus G proteins (Walker *et al.*, 1992), and appear to have arisen as a result of gene duplication (Wang and Walker, 1993). The G protein is the virion envelope glycoprotein that contains four major antigenic sites that are targets for neutralizing antibody (Cybinski *et al.*, 1990; Kongsuwan *et al.*, 1998) and has been shown to induce virus-specific neutralizing antibodies that confer passive protection against intracerebral infection of suckling mice (Cybinski *et al.*, 1990) and protect cattle against experimental intravenous BEFV challenge (Uren *et al.*, 1995; Hertig *et al.*, 1996). The GNS protein of BEFV has been detected in infected cells but does not occur in the virions (Walker *et al.*, 1992). It shares recognizable sequences homology with the G protein and structural characteristics including a high proportion of conserved cysteine, proline and glycine residues (Walker *et al.*, 1992). The GNS protein of BEFV does not induce antibodies that neutralize virus produced in mammalian or insect cells and does not induce a protective response in cattle (Hertig *et al.*, 1996). To date, the function of GNS in infected cells remains unknown. The M protein of rhabdoviruses is critical for virus assembly and budding. In the absence of other viral products, it is able to bud from cell surfaces in the form of lipid-enveloped and virus-like particles (Harty *et al.*, 2001). Amino acid sequence analysis of the nucleoprotein N of BEFV indicated a close relationship to that of ARV and similarity to other animal rhabdoviruses with higher overall homology to vesiculoviruses than to lyssaviruses (Walker *et al.*, 1994; Wang *et al.*, 1995). However, the BEFV and ARV genomes are more complex than other rhabdoviruses as each has two consecutive glycoprotein genes (Walker *et al.*, 1992; Wang and Walker, 1993).

12.2.2 Virus life cycle

To initiate a productive infection, all viruses must translocate their genome across their host-cell membrane (Smith and Helenius, 2004). Cell membrane is a barrier for outcoming sources, which include viruses. Virus penetration can take either by fusing with the plasma membrane directly to release viral capsids into cytosol or by endocytosis (Marsh and Helenius, 2006). Therefore, virus must bind to their host-cell surface, followed with signaling induction in order to penetrate into cells. The virus binding and entry of BEFV are similar to that of other membranes of the *Rhabdoviridae*, such as VSV. The glycoprotein G of VSV is involved in receptor recognition at the host-cell surface. It is suggested that G protein is distinct from both class I and II viral fusion proteins that had been already described (Gaudin, 2000; Roche *et al.*, 2008). After endocytosis of the virion, fusion is triggered by the low pH of the endosomal compartment and is mediated by the viral glycoprotein. G is an atypical fusion protein, as there is a pH-dependent equilibrium between its pre- and post-fusion conformations. In fact, the reversibility of the fusogenic low-pH-induced conformational change is crucial to allow G to be transported through the acidic compartments of the Golgi apparatus and to recover its native, pre-fusion state at the viral surface (Gaudin *et al.*, 1995).

Virus entry often uses multiple pathways and is cell-type specific, due to the component diversity in either membrane or organelles that might limit virus entry ability. Studies by Cheng *et al.* (2012) suggested that BEFV entry follows a clathrin-mediated and dynamin 2-dependent endocytosis pathway. BEFV infection also depends on endosomal acidification and on dynamin GTPase activity, which is a hallmark of clathrin-mediated endocytosis (Hinshaw, 2000). After entry, BEFV requires Rab5 and Rab7 for transport to early and late endosomes

for infection. Cheng *et al.* (2012) demonstrated that microtubules disruption impairs BEFV infectivity, because early endosomal transport requires microtubules and Rab5, suggesting that BEFV requires transport to early endosome for infection. Furthermore, data obtained from Cheng and colleagues also demonstrated that BEFV requires trafficking through early and late endosomes. In contrast to BEFV, VSV does not require late endosome function for infection (Sieczkarski and Whittaker, 2003).

12.3 Pathogenesis

12.3.1 Virus transmission

Bovine ephemeral fever (BEF) occurs frequently in Australia, Africa and Asia, including China, Taiwan and the Middle East (Wang *et al.*, 2001; Walker, 2005). The disease became enzootic in many countries, including Australia, China and Taiwan. In Asia, the Middle East and Africa, BEF occurs as epizootics, which originate in enzootic tropical areas and sweep north or south to sub-tropical and temperate zones. They are many possible transmission routes for arboviruses, such as wind and animal transport. The main routes of transmission between countries and continents have been studied (Aziz-Boaron *et al.*, 2012). Both winds and animal transport were found to be important factors in transboundary transmission of BEFV. For example, the Egyptian isolate grouped phylogenetically with the Taiwanese isolates, coinciding with results on importation of cattle from China to the Middle East in the year preceding the isolation of the Egyptian isolates (Aziz-Boaron *et al.*, 2012).

BEF is most prevalent during rainy seasons when flying insects are numerous, and the spread may be affected by wind movement. The causative virus is transmitted by haematophagous biting insects, probably mosquitoes or midges that can be carried on the wind allowing rapid spread of the disease. It was reported that BEFV can be isolated from various species of mosquitoes and culicoides (Davies and Walker, 1974; Blackburn *et al.*, 1985). Studies on models for arbovirus dispersion by winds may help in quantifying the risk of BEFV transmission in Australia, Africa and Asia, as well as its invasion to other geographical locations, including Europe (Hendrickx *et al.*, 2008; Ducheyne *et al.*, 2011).

12.3.2 Pathology

BEF is an inflammatory disease and is characterized by some common pathological lesions, including serofibrinous polysinovitis, polyarthritis, polytendovaginitis, cellulitis and focal necrosis of skeletal muscles. The lungs may show fatty oedema and lymph nodes are oedematous. Lesions in the upper cervical region of the spinal cord were also seen (Hill and Schultz, 1977). Histopathology reveals neutrophilia, leukocytosis and high fibrinogen level. A reduction in erythrocyte number in the early part of the infection followed by a larger fall corresponds to haemosiderosis of lymph node and spleen. Lesions have also been found in venules and capillaries in tendon sheath, synovial membranes, muscle, fascia and endothelium, along with perivascular neutrophilic infiltration, focal or complete necrosis of vessel walls, thrombosis and perivascular fibrosis (Basson *et al.*, 1970).

12.3.3 Clinical outcome

BEF causes an acute febrile disease in cattle and water buffalo. Neutralizing antibodies have also been found in other species of African wildlife (Davies *et al.*, 1975). BEF is widespread throughout Asia, Australia, Africa and the Middle East, and is commonly known as ‘ephemeral fever’ or ‘three-day stiffness sickness’ because of the immobilization of infected animals for 3–5 days following the height of viraemia and fever (Bevan, 1912; St George, 1994). Accumulating evidence indicates that clinical signs of BEF, which include bi-phasic fever, anorexia, muscle stiffness, ocular and nasal

discharge, ruminal stasis and recumbency, are due primarily to a vascular inflammatory response (Nandi and Negi, 1999; Walker, 2005). Its economic effect is mainly due to mortality and severe reduction in milk production in dairy herds, loss of draught animals at the time of harvest and loss of condition in beef cattle. The reduction in milk production ranges from 34% to 95%, with an average of 46%. The milk yield did not return to pre-illness level on convalescence. There is some loss from abortion in females (Theodoridis *et al.*, 1973) and males may suffer from temporary infertility (Parsonson and Snowdown, 1973). Although recovery may be complete, a permanent drop in milk production in cows and reduced fertility in bulls often occurs, resulting in heavy economic losses (St George, 1994). In addition, BEFV poses a severe threat to both national and international trade of animals. Many countries require imported cattle and buffalo to be free from BEF-neutralizing antibodies. It is expensive to keep bulls whose semen is to be exported in insect proof areas and to monitor the evidence of BEFV infection continuously (Walker and Cybinski, 1989).

12.4 Molecular Pathogenesis

12.4.1 Virus entry

Endocytosis is required for a number of cellular functions, including pathogen entry, antigen presentation, nutrient uptake, drug delivery, neurotransmission, membrane receptor recycling, cell adhesion and migration, cell polarity, mitosis, cell growth and cell differentiation. Many viruses have evolved to utilize endocytosis to enter their host cells after initial binding of virions to receptor(s) on the cell membrane. After attachment to their host-cell surface receptor(s), internalization usually follows directly from the plasma membrane or via endocytosis. To date, a number of different routes of endocytosis used by viruses have been demonstrated. The best understood endocytic pathways include clathrin-dependent and lipid raft/caveolae-dependent entry pathways. Clathrin-mediated endocytosis is a major endocytic pathway from the plasma membrane to early endosomes. In clathrin-mediated endocytosis, clathrin is assembled on the inside face of the plasma membrane to form a characteristic coated pit (CCP) and in response to receptor-mediated internalization signals. Once assembled, CCPs pinch off from the cell membrane and mature into clathrin-coated vesicles (Keen, 1990), which then transport the cargo into endosomes. Dynamin is critical for clathrin- or caveolae-mediated endocytosis and phagocytosis, but it is not necessary for macropinocytosis (Henley *et al.*, 1998; Huang *et al.*, 2011). A number of viruses have been discovered to enter their host cells through the clathrin-dependent endocytosis pathway (Helenius *et al.*, 1980; Sieczkarski and Whittaker, 2005; Sun *et al.*, 2005). Many other viruses have also been reported to enter their host cells through the caveolae-dependent pathway (Bousarghin *et al.*, 2003; Huang *et al.*, 2011).

The specific cell pathways involved in cell entry of BEFV have been determined (Cheng *et al.*, 2012). By using a fluorescently lipophilic dye, 3,30-dilinoleyloxacarbocyanine perchlorate (DiO) labelled-BEFV, Cheng *et al.* (2012) demonstrated that clathrin-mediated and dynamin 2-dependent endocytosis in a pH-dependent manner is an important avenue of BEFV entry. After endocytosis, Rab 5 and Rab 7 were activated and involved in early and late endosomes that are required for endocytosis and subsequent infection in cells. Treatment of BEFV-infected cells with nocodazole significantly decreased the viral protein synthesis and viral yield, suggesting that microtubules play a critical role in BEFV productive infection likely by mediating trafficking of BEFV-containing endosomes.

12.4.2 Virus–host interactions and virus-induced apoptosis

Evidence indicates that infection by rhabdoviruses such as VSV, spring viraemia of carp virus (SVCV) and the rabies virus

results in apoptotic cell death (Licata and Harty, 2003). The mechanism of VSV-induced apoptosis requires caspase cascade activation and suppression of host gene expression, and its matrix protein plays a major role in regulating the apoptotic process (Desforges *et al.*, 2002; Hobbs *et al.*, 2003; Gaddy and Lyles, 2005). Apoptosis induction by BEFV in several cell lines has also been demonstrated (Chang *et al.*, 2004). In order to determine the step in viral life cycle at which apoptosis of infected cells is triggered, chemical and physical agents were usually utilized to inhibit viral infection. Previous findings suggested that treatment of BHK-21 infected cells with ammonium chloride (NH_4Cl) or cells infected with UV-inactivated BEFV was seen to block virus apoptosis induction, indicating that virus uncoating and gene expression are required for the induction of apoptosis (Lin *et al.*, 2009). By using soluble death receptors Fc:Fas chimera to block Fas signaling, apoptosis induced by BEFV was inhibited in cells. BEFV can replicate and induce apoptosis in Vero and Madin-Darby bovine kidney (MDBK) cells. A kinetic study showed a higher efficiency of replication and a greater apoptosis induction ability of BEFV in Vero cells (Chen *et al.*, 2010). BEFV infection can result in the Fas-dependent activation of caspase 8 and cleavage of Bid in its host cells. Furthermore, Fas-mediated BEFV-induced apoptosis was demonstrated to be suppressed by the overexpression of Bcl-2 or by treatment with caspase inhibitors and soluble death receptors Fc:Fas chimera. Therefore, Lin *et al.* (2009) further suggested that BEFV-induced apoptosis requires activation of Fas and mitochondrion-mediated caspase-dependent pathways. A Src-dependent JNK signaling pathway triggered by BEFV was also demonstrated to promote BEFV-induced apoptosis (Chen *et al.*, 2010). In BEFV-infected Vero and MDBK cells, BEFV directly induces Src Y418 phosphorylation and JNK phosphorylation and kinase activity, which were inhibited specifically by Src and JNK inhibitors (SU6656 and SP600125), respectively. The caspase cascade and its downstream effectors, poly(ADP-ribose) polymerase (PARP) and DFF45, were activated simultaneously upon BEFV infection (Chen *et al.*, 2010). The cytopathic effect (CPE) is caused by apoptosis, which is damage to host cells caused by virus infection, and leads to visible morphologic changes. Different types of cells in a host individual may respond differently to a viral infection. Chen *et al.* (2010) demonstrated that viral protein synthesis can be detected 12 hours after BEFV infection at an early stage of CPE, and the process can last for more than 12 hours post-infection (Walker *et al.*, 1991). Therefore, virus-induced apoptosis reveals a way to reduce the CPE, thereby reducing the mortality caused by BEFV infection.

Several viruses rely on activation of the PI3K-Akt pathway for efficient replication or long-term persistence. Similar to findings with other non-segmented, negative-sense RNA viruses (NNSVs) (Sun *et al.*, 2008), inhibition of Akt through the use of Akt inhibitors had negative effects on BEFV replication, suggesting that activation of Akt is required for BEFV propagation (Ji *et al.*, 2011). Interestingly, suppression of PI3K or mTOR complex 1 (mTORC1) through the use of inhibitors (wortmannin, LY294002 and rapamycin) increased BEFV replication (Ji *et al.*, 2011). Some viruses, such as hepatitis B virus (HBV) and hepatitis C virus (HCV), which are persistent viruses, can activate PI3K-Akt-mTOR signaling to promote cell survival and long-term infection. Inhibition of PI3K, Akt or mTOR slightly up-regulates replication of these two viruses (Mannova and Beretta, 2005; Guo *et al.*, 2007). Unlike HBV and HCV, BEFV possesses a different survival strategy, as seen from its reliance on Akt for efficient replication but not PI3K and mTORC. BEFV might maintain Akt activity to slow down cell death and prolong viral infection.

12.4.3 Immune response

Vertebrates acquire immunity to rhabdoviruses in much the same manner as they do in

other acute viral infection. Primary infection results in a humoral immune response developing within a week after first exposure. Cellular immune response also occurs after infection. Antibodies created during the humoral immune response are directed to two major antigens, the group-specific N protein and type-specific G protein. The type-specific G protein of BEFV is the viral antigen that gives rise to and reacts with neutralizing antibodies (Cybinski *et al.*, 1990; Kongsuwan *et al.*, 1998). The use of hybridoma cells that secrete monoclonal antibody has been extensively exploited to investigate the epitopes on the G protein (Cybinski *et al.*, 1990; Kongsuwan *et al.*, 1998). A number of BEFV isolates isolated in Australia between 1956 and 1992 were examined and revealed sequence conservation in most neutralization sites on the G protein. However, epitope shifts have been detected in the major conformational site G3 on the BEFV G protein (Walker, 2005). BEFV isolates from China and Taiwan are closely related to Australian isolates, but some variations have been uncovered (Walker, 2005; Hsieh *et al.*, 2006). It is interesting to note that Aziz-Boaron *et al.* (2012) demonstrated that BEFVs circulating in Israel and Turkey are closely related phlyogenetically and differ from the Australian and the East Asian isolates, including the Egyptian isolates. The Egyptian isolates from 2005 have been observed to be similar to the 2004 Taiwanese isolates (Hsieh *et al.*, 2005).

To date, several forms of live-attenuated, inactivated and recombinant vaccines have been reported but with variable efficacy and durability of protection. The G protein of BEFV is a highly effective vaccine antigen, either as a purified subunit or expressed from recombinant viral vectors (Hertig *et al.*, 1996; Walker, 2005; Johal *et al.*, 2008). Natural BEFV infection induces a strong neutralizing antibody response and infection usually induces durable immunity. It is necessary to replace current live-attenuated virus vaccines that are sensitive to heat, especially in Africa where maintenance of a continuous cold-chain is quite difficult.

12.5 Diagnosis

During major outbreaks, involving entire herds, the diagnosis is primarily based on clinical outcomes and history of the outbreak. However, individual cases are difficult to diagnose (Uren *et al.*, 1992). A differential leukocyte count on the blood smear provides the most rapid supporting evidence for field diagnosis, but a high percentage of neutrophils with many immature forms are not pathognomonic of ephemeral fever. Sporadic cases, or cases occurring early in a possible epidemic, can be confirmed by virus isolation and identification (Tzipori, 1975) or serology to detect specific antibodies in paired serum samples (Nandi and Negi, 1999). There are several immunological methods established for detection of specific antibodies to BEFV, including a blocking ELISA (Zakrzewiski *et al.*, 1992; Hsieh *et al.*, 2005). BEF virus antigen can be detected in blood leukocyte films prepared from feverish animals by immunofluorescent test (Zaghawa *et al.*, 2002). However, a sensitive, reliable and quantitative technique for serological diagnosis of BEFV is currently lacking. Currently, virus isolation seems to be the standard method for BEF diagnosis.

In recent years, nucleic acid detection has become a standard technique for monitoring virus infection. Molecular diagnostic assays have been reported to detect the genome of BEFV (Table 12.1). A real-time RT-PCR assay, targeting the glycoprotein (G) gene of BEFV, has been developed by Stram *et al.* (2011). This assay appears to be sensitive and has a detection limit of 10 copies of BEFV genome in a clinical sample. Using RT-PCR, a primer designed to amplify 438 nucleotide fragments from the G gene, allowed specific amplification of BEFV-cell culture isolates collected from Egypt and Japan. Zaghloul *et al.* (2012) used same gene spot successfully in Dot-blot hybridization assay. A rapid, sensitive and specific assay has been developed to detect and quantify the G protein-encoding gene of BEFV known as 'a reliable assay protocol for identification of diseases (RAPID)-bioactive amplification with probing (BAP) assay' (Hsieh *et al.*, 2005). This technique combines a nested

Table 12.1. Features of the available PCR formats for the detection of BEFV.

Assay type	Forward primer (5′–3′)	Reverse primer (5′–3′)	Probe (5′–3′)	Reference
Real-time PCR	GAGATCAAATGTCCA CAACGTTTAA	AATGTTCATCCTTTGC AAGATTATGA	AATTATCACTT CAAGCCC	Stram *et al.* (2005)
RT-PCR	TACAACAGCAGATAA AAC	CATTATGGGATAGGA TCC 3′	–	Zaghloul *et al.* (2012)
LAMP	Primer name	Primer sequence (5′–3′)	–	Zheng *et al.* (2011)
RT-LAMP amplification	F3	TGACAGTAAGACAAA GGGATGT	–	
	B3	CATCCTCGAAAATGA TGCCAT	–	
	FIP: F2	GGACATGGATACCAG ATGTGAG-	–	
	FIP: F1c	GGACTTAACTGTAATG CATTCCCA	–	
	BIP: F2	TCCACAAAACGTAGAC AGACAT-	–	
	BIP: F1c	ATGAGACAGAGAGATT GTGGGA	–	
RT-PCR amplification	420F	AGAGCTTGGTGTGAA TAC	–	
	420R	CCAACCTACAACAGCA GATA	–	

PCR and magnetic bead-based DNA probing assay. By using this technique, a minimum of 1 copy/μl of the BEFV plasmid DNA could be detected. Furthermore, Hsieh *et al.* (2005) have compared the sensitivity of the RAPID-BAP assay, real-time RT-PCR, and conventional RT-PCR in the detection of clinical blood samples suspected to have BEFV infections, and the results revealed that the RAPID-BAP assay was more sensitive than the conventional RT-PCR and real-time RT-PCR assays for the detection of BEFV. The novel RAPID-BAP assay is an excellent diagnostic tool with high sensitivity and specificity. Another assay, reverse transcription loop-mediated isothermal amplification (RT-LAMP), again targeting the G gene of BEFV, has also been developed. The advantage of RT-LAMP is that no specialist equipment is required because there is no requirement for thermal cycling (Zheng *et al.*, 2011). However, a range of specialist apparatus is now available specifically for application with RT-LAMP such as lateral flow devices and turbidometers. Given the above, current available assays could not quickly and completely detect BEFV. A high-speed assay with high specificity and sensitivity to detect BEFV from available samples is urgently needed.

References

Aziz-Boaron, O., Klausner, Z. Hasoksuz, M., Shenkar, J., Gafni, O., Gelman, B., David, D. and Klement, E. (2012) Circulation of bovine ephemeral fever in the Middle East: strong evidence for transmission by winds and animal transport. *Veterinary Microbiology* 158, 300–307.

Basson, P.A., Pienaar, J.G. and Van der Westhuizen, B. (1970) The pathology of ephemeral fever: a study of the experimental disease in cattle. *Journal of the South African Veterinary Association* 40, 385–397.

Bevan, L.E.W. (1912) Ephemeral fever or three days' sickness of cattle. *Veterinary Journal* 68, 458–461.

Bishop, D.H.L., Ghabrial, S.A., Jarvis, A.W., Martelli, G.P., Mayo, M.A. and Summers, M.D. (2009) *Virus Taxonomy: Sixth Report of the International Committee on Taxonomy of Viruses*. Springer, New York, pp. 384–387.

Blackburn, N.K., Searle, L. and Phelps, R.J. (1985) Viruses isolated from cullcoides (Diptera: *Ceratopogonidae*) caught at the veterinary research farm, Mazowe, Zimbabwe. *Journal of Entomology Society of South Africa* 48, 331.

Bousarghin, L., Touze, A., Sizaret, P.Y. and Coursaget, P. (2003) Human papillomavirus types 16, 31, and 58 use different endocytosis pathways to enter cells. *Journal of Virology* 77, 3846–3850.

Calisher, C.H., Karabatsos, N., Zeller, H., Digoutte, J.P., Tesh, R.B., Shope, R.E., Travassos da Rosa, A.P.A. and St George, T.D. (1989) Antigenic relationships among rhabdoviruses from vertebrates and haematophagous arthropods. *Intervirology* 49, 241–257.

Chang, C.J., Shih, W.L., Yu, F.L., Liao, M.H. and Liu, H.J. (2004) Apoptosis induced by bovine ephemeral fever virus. *Journal of Virological Methods* 22, 165–170.

Chen, C.Y., Chan, C.Y., Liu, H.J., Liao, M.H., Chang, C.I., Hsu, J.L. and Shih, W.L. (2010) Apoptosis induction in BEFV-infected Vero and MDBK cells through Src-dependent JNK activation regulates caspase-3 and mitochondria pathways. *Veterinary Research* 41, 15.

Cheng, C.Y., Shih, W.L., Huang, W.R., Chi, P.I., Wu, M.H. and Liu, H.J. (2012) Bovine ephemeral fever virus uses a clathrin-mediated and dynamin 2-dependent endocytosis pathway that requires Rab5 and Rab7 as well as microtubules. *Journal of Virology* 86, 1–9.

Cybinski, D.H., Walker, P.J., Byrne, K.A. and Zakrzewsk, H. (1990) Mapping of antigenic sites on the bovine ephemeral fever virus glycoprotein using monoclonal antibodies. *Journal of General Virology* 7, 2065–2072.

Davies, F.G. and Walker, A. (1974) The isolation of ephemeral fever virus from cattle and culicoides midges in Kenya. *Veterinary Record* 95, 63–64.

Davies, F.G., Shaw, T. and Ochieng, P. (1975) Observations on the epidemiology of ephemeral fever in Kenya. *Journal of Hygiene* 75, 231–235.

Della-Porta, A.J. and Brown, F. (1979) The physico-chemical characterization of bovine ephemeral fever virus as a member of the family *Rhabdoviridae*. *Journal of General Virology* 44, 99–12.

Desforges, M., Despars, G., Berard, S., Gosselin, M., McKenzie, M.O. and Lyles, D.S. (2002) Matrix protein mutations contribute to inefficient induction of apoptosis leading to persistent infection of human neural cells by vesicular stomatitis virus. *Virology* 295, 63–73.

Dhillon, J. (1996) Molecular study of bovine ephemeral fever virus proteins. PhD dissertation. University of Queensland, Queensland, Australia.

Ducheyne, E., Lange, M., Van der Stede, Y., Meroc, E., Durand, B. and Hendrickx, G. (2011) A stochastic predictive model for the natural spread of bluetongue. *Preventive Veterinary Medicine* 99, 48–59.

Gaddy, D.F. and Lyles, D.S. (2005) Vesicular stomatitis viruses expressing wild-type or mutant M proteins activate apoptosis through distinct pathways. *Journal of Virology* 79, 4170–4179.

Gaudin, Y. (2000) Reversibility in fusion protein conformational changes. The intriguing case of rhabdovirus-induced membrane fusion. *Subcellular Biochemistry* 34, 379–408.

Gaudin, Y., Tuffereau, C., Durrer, P., Flamand, A. and Ruigrok, R.W. (1995) Biological function of the low-pH, fusion-inactive conformation of rabies virus glycoprotein (G): G is transported in a fusion-inactive state-like conformation. *Journal of Virology* 69, 5528–5534.

Guo, H., Zhou, T., Jiang, D., Cuconati, A., Xiao, G.H., Block, T.M. and Guo, J.T. (2007) Regulation of hepatitis B virus replication by the phosphatidylinositol 3-kinase-Akt signal transduction pathway. *Journal of Virology* 81, 10,072–10,080.

Harty, R.N., Brown, M.E., McGettigan, J.P., Wang, G., Jayakar, H.R., Huibregtse, J.M., Whitt, M.A. and Schnell, M.J. (2001) Rhabdoviruses and the cellular ubiquitin-proteasome system: a budding interaction. *Journal of Virology* 75, 10,623–10,629.

Helenius, A., Kartenbeck, J., Simons, K. and Fries, E. (1980) On the entry of Semliki forest virus into BHK-21 cells. *Journal of Cell Biology* 84, 404–420.

Hendrickx, G., Gilbert, M., Staubach, C., Elbers, A., Mintiens, K., Gerbier, G. and Ducheyne, E. (2008) A wind density model to quantify the airborne spread of *Culicoides* species during north-western Europe bluetongue epidemic, 2006. *Preventive Veterinary Medicine* 7, 162–181.

Henley, J.R., Krueger, E.W., Oswald, B.J. and McNiven, M.A. (1998) Dynamin-mediated internalization of caveolae. *Journal of Cell Biology* 141, 85–99.

Hertig, C., Pye, A.D., Hyatt, A.D., Davis, S.S., McWilliam, S.M., Heine, H.G., Walker, P.J. and Boyle, D.B. (1996) Vaccinia virus-expressed bovine ephemeral fever virus G but not G_{NS} glycoprotein induces neutralizing antibodies and protects against experimental infection. *Journal of General Virology* 77, 631–640.

Hill, M.W.M. and Schultz, L. (1977) Ataxia and paralysis associated with bovine ephemeral fever infection. *Australian Veterinary Journal* 53, 217–221.

Hinshaw, J.E. (2000) Dynamin and its role in membrane fission. *Annual Review of Cell and Developmental Biology* 16, 483–519.

Hobbs, J.A., Hommel-Berrey, G. and Brahmi, Z. (2003) Requirement of caspase-3 for efficient apoptosis induction and caspase-7 activation but not viral replication or cell rounding in cells infected with vesicular stomatitis virus. *Human Immunology* 64, 82–92.

Hsieh, Y.C., Chen, S.H., Chou, C.S., Hsiao, H.W., Chen, S.Z., Lee, Y.F. and Liu, H.J. (2005). Development of a reliable assay protocol for identification of diseases (RAPID)-bioactive amplification with probing (BAP) for detection of bovine ephemeral fever virus. *Journal of Virological Methods* 129, 75–82.

Hsieh, Y.C., Chen, S.H., Chou, C.C., Ting, L.J., Itakura, C. and Wang, F.I. (2005) Bovine ephemeral fever in Taiwan (2001–2002). *Journal of Veterinary Medicine and Sciences* 67, 411–416.

Hsieh, Y.C., Wang, S.Y., Lee, Y.F., Chen, S.H., Mak, P.O. and Chu, C.Y. (2006) DNA sequence analysis of glycoprotein G gene of bovine ephemeral fever virus and development of a double oil emulsion vaccine against bovine ephemeral fever. *Journal of Veterinary Medicine and Science* 68, 543–548.

Huang, W.R., Wang, Y.C., Chi, P.I., Wang, L., Wang, C.Y., Lin, C.H. and Liu, H.J. (2011) Cell entry of avian reovirus follows a caveolin-1-mediated and dynamin 2-dependent endocytic pathway that requires activation of p38 MAPK and Src signaling pathways as well as microtubules and small GTPase Rab5 protein. *Journal of Biological Chemistry* 286, 30,780–30,794.

Ji, W.T., Ying, C., Wang, L., Lin, F.L., Liao, M.H., Shih, W.L. and Liu, H.J. (2011) Inhibitors of phosphatidylinositol 3-kinase and mTOR but not Akt enhance replication of bovine ephemeral fever virus. *Veterinary Journal* 187, 119–123.

Johal, J., Gresty, K., Kongsuwan, K. and Walker, P.J. (2008) Antigenic characterization of bovine ephemeral fever rhabdovirus G and GNS glycoproteins expressed from recombinant baculoviruses. *Archives of Virology* 153, 1657–1665.

Keen, J.H. (1990) Clathrin and associated assembly and disassembly proteins. *Annual Review of Biochemistry* 59, 415–438.

Kongsuwan, K., Cybinski, D.H., Cooper, J. and Walker, P.J. (1998) Location of neutralizing epitopes on the G protein of bovine ephemeral fever rhabdovirus. *Journal of General Virology* 79, 2573–2581.

Licata, J.M. and Harty, R.N. (2003) Rhabdoviruses and apoptosis. *International Review of Immunology* 22, 451–476.

Lin, C.H., Shih, W.L., Lin, F.L., Hsieh, Y.C., Kuo, Y.R., Liao, M.H. and Liu, H.J. (2009) Bovine ephemeral fever virus-induced apoptosis requires virus gene expression and activation of Fas and mitochondrial signaling pathway. *Apoptosis* 14, 864–877.

Mannova, P. and Beretta, L. (2005) Activation of the N-Ras-PI3K-Akt-mTOR pathway by hepatitis C virus: control of cell survival and viral replication. *Journal of Virology* 79, 8742–8749.

Marsh, M. and Helenius, A. (2006) Virus entry: open sesame. *Cell* 124, 729–740.

McWilliam, S.M., Kongsuwan, K., Cowley, J.A., Byrne, K.A. and Walker, P.J. (1997) Genome organization and transcription strategy in the complex GNS–L intergenic region of bovine ephemeral fever rhabdovirus. *Journal of General Virology* 78, 1309–1317.

Murphy, F.A., Taylor, W.P., Mims, C.A. and Whitfield, S.G. (1972) Bovine phemeral fever in cell culture and mice. *Archive fuer die Gesamte Virusforschung* 38, 234–249.

Nandi, S. and Negi, B.S. (1999) Bovine ephemeral fever: a review. *Comparative Immunology, Microbiology and Infectious Diseases* 22, 81–91.

Parsonson, I.M. and Snowdown, W.A. (1973) Experimental infection of pregnant cattle with ephemeral fever virus. *Australian Veterinary Journal* 50, 335–337.

Roche, S., Albertini, A.A., Lepault, J., Bressanelli, S. and Gaudin, Y. (2008) Structures of vesicular stomatitis virus glycoprotein: membrane fusion revisited. *Cellular and Molecular Life Science* 65, 1716–1728.

Sieczkarski, S.B. and Whittaker, G.R. (2003) Differential requirements of Rab5 and Rab7 for endocytosis of influenza and other enveloped viruses. *Traffic* 4, 333–343.

Sieczkarski, S.B. and Whittaker, G.R. (2005) Characterization of the host cell entry of filamentous influenza virus. *Archives of Virology* 150, 1783–1796.

Smith, A.E. and Helenius, A. (2004) How viruses enter animal cells. *Science* 304, 237–242.

St George, T.D. (1990) Bovine ephemeral fever virus. In: Dinner, Z. and Morein, B. (eds) *Virus Infections of Vertebrates*, vol. 3. Elsevier, Amsterdam, pp. 404–415.

St George, T.D. (1994) Bovine ephemeral fever. In: Coetzer, J.A.W., Thomson, G.R. and Tustin, R.C. (eds) *Infectious Diseases of Livestock with Special Reference to Southern Africa*, vol. 1. Oxford University Press, Cape Town, pp. 553–562.

Stram, Y., Kuznetzova, L., Levin, A., Yadin, H. and Rubinstein-Giuni, M. (2011) A real-time RT-quantative(q) PCR for the detection of bovine ephemeral fever virus. *Journal of Virological Methods* 130, 1–6.

Sun, M., Fuentes, S.M., Timani, K., Sun, D., Murphy, C., Lin, Y., August, A., Teng, M.N. and He, B. (2008) Akt plays a critical role in replication of nonsegmented negative-stranded RNA viruses. *Journal of Virology* 82, 105–114.

Sun, X., Yau, V.K., Briggs, B.J. and Whittaker, G.R. (2005) Role of clathrin-mediated endocytosis during vesicular stomatitis virus entry into host cells. *Virology* 338, 53–60.

Theodoridis, A., Giesecke, W.H. and Du Toit, I.J. (1973) Effects of ephemeral fever on milk production and reproduction in dairy cattle. *Onderstepoort Journal of Veterinary Research* 40, 83–91.

Tordo, N., Benmansour, A., Calisher, C., Dietzgen, R.G., Fang, R.X., Jackson, A.O., Kurath, G., Nadin-Davis, S., Tesh, R.B. and Walker, P.J. (2005) Rhabdoviridae. In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U. and Ball, L.A. (eds) *Virus taxonomy, VIIIth report of the ICTV*. Elsevier/Academic Press, London, pp. 623–644.

Tzipori, S. (1975) Plaque assay and characteristics of strains of bovine ephemeral fever virus in Vero cells. *British Veterinary Journal* 131, 720–726.

Uren, M.F., George, M.F. and Murphy, G.M. (1992) Studies on the pathogenesis of bovine ephemeral fever in experimental cattle. III. Virological biochemical data. *Veterinary Microbiology* 30, 297–307.

Walker, P.J. (2005) Bovine ephemeral fever in Australia and the world. *Current Topics in Microbiology and Immunology* 292, 57–80.

Walker, P.J. and Cybinski, D.H. (1989) Bovine ephemeral fever and rhabdoviruses endemic to Australia. *Australian Veterinary Journal* 66, 398–400.

Walker, P.J., Byrne, K.A., Cybinski, D.H., Doolan, D.L. and Wang, Y.H. (1991) Proteins of bovine ephemeral fever virus. *Journal of General Virology* 72, 67–74.

Walker, P.J., Byrne, K.A., Riding, G.A., Cowley, J.A., Wang, Y. and McWilliam, S. (1992) The genome of bovine ephemeral fever rhabdovirus contains two related glycoprotein genes. *Virology* 191, 49–61.

Walker, P.J., Wang, Y., Cowley, J.A., McWilliam, S.M. and Prehaud, C.J. (1994) Structural and antigenic analysis of the nucleoprotein of bovine ephemeral fever rhabdovirus. *Journal of General Virology* 75, 1889–1899.

Wang, F.I., Hsu, A.M. and Huang, K.J. (2001) Bovine ephemeral fever in Taiwan. *Journal of Veterinary Diagnosis and Investigation* 13, 462–467.

Wang, Y. and Walker, P.J. (1993) Adelaide River rhabdovirus expresses consecutive glycoprotein genes as polycistronic mRNAs: new evidence of gene duplication as an evolutionary process. *Virology* 195, 719–731.

Wang, Y., Cowley, J.A. and Walker, P.J. (1995) Adelaide River virus nucleoprotein gene: analysis of phylogenetic relationships of ephemeroviruses and other rhabdoviruses. *Journal of General Virology* 76, 995–999.

Wunner, W.H., Calisher, C.H., Dietzgen, R.G., Jackson, A.O., Kitajima, E.W., Lafon, M.F., Leong, J.C., Nichol, S.T., Peters, D., Smith, J.S. and Walker, P.J. (1995) The rhabdoviridae. In: Murphy, F.A., Fauquet, C.M., Bishop, D.H.L. *et al*. (eds) *Virus Taxonomy. Sixth Report of the International Committee on Taxonomy of Viruses*. Springer, New York, pp. 288–293.

Zaghawa, A., Khalil, S.A., Hassan, H.Y. and Al-Gabary, M.H. (2002) Diagnostic value of the antigenic relationship between bovine ephemeral fever and rabies viruses. 2nd Scientific Veterinary Medicine Congress. Faculty of Veterinary Medicine, Menufia University, Sadat City, Egypt, 23–24 April 2002.

Zaghloul, A.H., Mahmoud, A., Hassan, H.Y., Hemeida, A.A., Nayel, M.A. and Zaghawa, A.A. (2012) Establishment of dot-blot hybridization for diagnosis of bovine ephemeral fever virus in Egypt. *International Journal of Virology* 8, 271–278.

Zakrzewski, H., Cybinski, D.H. and Walker, P.J. (1992) A blocking ELISA for the detection of specific antibodies to bovine ephemeral fever virus. *Journal of Immunological Methods* 151, 289–297.

Zheng, F., Lin, G., Zhou, J., Wang, G., Cao, X., Gong, X. and Qui, C. (2011) A reverse-transcription, loop-mediated isothermal amplification assay for detection of bovine ephemeral fever virus in the blood of infected cattle. *Journal of Virological Methods* 171, 306–309.

13 Rabies Virus

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13.1 Introduction

Rabies is a major zoonotic disease, which remains a serious world public health problem. It is one of the most recognizable zoonosis and has been well known for more than 4300 years (Takayama, 2005). While rabies has been controlled throughout most of the developed world, it remains a significant burden in developing countries, causing a large number of animal and human deaths (Wilde and Lumlertdacha, 2011). According to WHO estimates, 55,000 human deaths are reported worldwide and more than 10 million people undergo post-exposure prophylaxis every year. Most human cases occur in the developing countries of Asia and Africa, where canine rabies is endemic (Fu, 1997). In more developed countries, human rabies has dramatically declined over the past 60 years as a direct consequence of routine vaccination of pet animals. However, rabies in wildlife has emerged as a major threat. Despite extensive investigations over more than 100 years, the pathogenetic mechanisms by which infection of street rabies virus (RABV) results in neurological diseases and death in humans are not well understood. Many papers have summarized studies on rabies pathogenesis (Dean *et al.*, 1963; Ritossa and D'Agostino, 1967; Dierks, 1979; Dietzschold *et al.*, 2005, 2008; Hooper, 2005; Jackson, 2006). In this chapter, we briefly describe our current understanding of rabies pathogenesis, the use of RABV as a neuronal tracer and molecular diagnosis for the disease.

RABV has a bullet-shaped structure of about 75 nm×200 nm and this RNA virus contains a negative-stranded RNA genome of about 12 kb, which encodes five proteins in the order of nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the RNA-dependent RNA polymerase (RdRp, also termed L). The N protein encapsulates the newly synthesized RNA, switching the virus from transcription to replication (Lawson *et al.*, 1991). The P protein is important not only for viral transcription and replication but also for interaction with cellular proteins during axoplasmic transport (Lawson *et al.*, 1991). The M protein is located beneath the viral membrane and bridges the nucleocapsid and the lipid bilayer and is involved in viral assembly and budding (Lenard, 1996). The G gene produces a single transmembrane G protein, which is assembled as a trimeric spike (Lawson *et al.*, 1991). This G protein is responsible for the initial binding during infection of susceptible cells (Lawson *et al.*, 1991). The L gene encodes a polymerase for viral RNA transcription and replication.

RABV is a highly neurotropic virus that spreads along neural pathways and invades

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the central nervous system (CNS), where it causes an acute infection. Most studies on rabies pathogenesis have used experimental animal models infected with laboratory-adapted strains, although the events in these models may not closely mimic the disease under natural conditions (Morimoto *et al.*, 1998, Suja *et al.*, 2011). There are a number of sequential steps that occur after peripheral inoculation of the virus from an animal bite, which is the most common mechanism of transmission (Jackson, 2002, 2006). The steps include replication in peripheral tissues, spread along peripheral nerves and the spinal cord to the brain, dissemination within the CNS, and centrifugal spread from the CNS along nerves to various organs, including the salivary glands (Fig. 13.1).

13.2 Earliest Events at the Site of Exposure

Under natural conditions, humans and animals may experience long and variable incubation periods following a bite exposure, usually lasting for 20 to 90 days (Smith *et al.*, 1991). However, incubation periods shorter than 20 days and longer than 90 days have been reported (Smith *et al.*, 1991). The biologic bases for the variable incubation periods are unclear, but a number of factors may be responsible, including the density of RABV receptors in affected tissues, the degree of innervation in tissues in different anatomical locations, the quantity of virus inoculated and the biological properties of RABV variant (Ray *et al.*, 1995). Many studies have been performed to determine the events during the incubation period and viral spread following the bite exposure. For example, Baer *et al.* (1968) performed experiments involving amputation of the tail or leg of an animal proximal to the site of inoculation or neurectomy of sciatic nerve. The results clearly demonstrated that RABV moves along peripheral nerves to the CNS in a time-dependent manner. Studies using street RABV supported the idea that the virus remains at or near the site of entry for most of the long incubation period (Baer and Cleary, 1972).

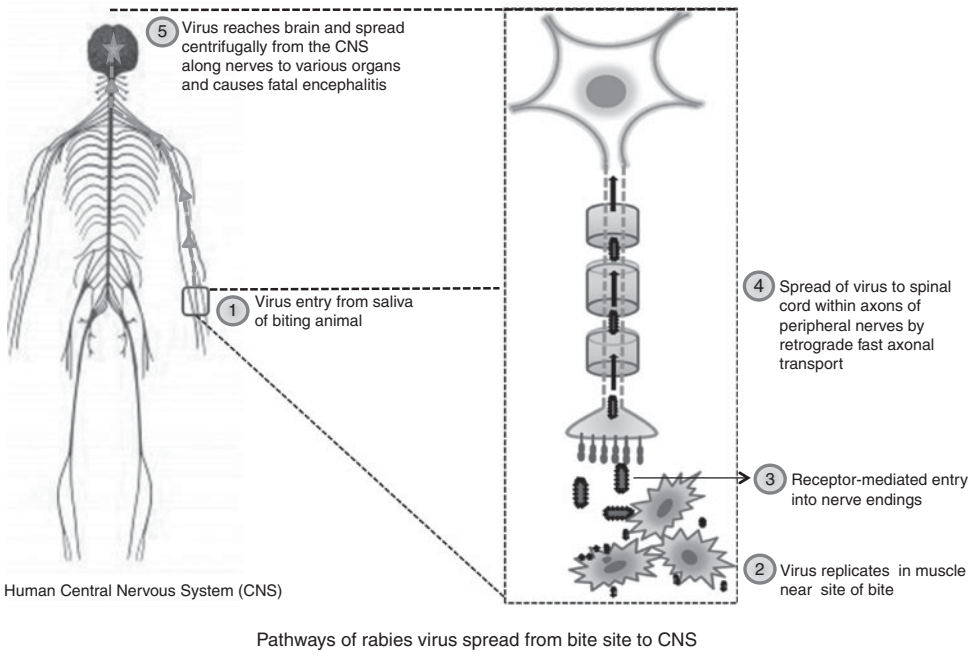

Fig. 13.1. The sequential steps involved in RABV spread from the bite site to the CNS.

There has been speculation that RABV may infect, replicate and subsequently persist *in vivo* in macrophages (Ray *et al.*, 1995), but the extent of viral replication in macrophages at the site of infection/injection has not yet been clearly demonstrated in animal models. The best experimental example to date was in striped skunks using a Canadian isolate of street RABV by Charlton *et al.* (1997), where they showed the presence of viral genomic RNA in the inoculated muscle, but not in either spinal ganglia or the spinal cord for up to 62–64 days. Immunohistochemistry supported infection of extrafusal muscle fibres and occasional fibrocytes at the site of inoculation. However, the importance of RABV infecting muscle fibres to gain access to peripheral nervous system and its relevance in pathogenicity is still being debated. Bats produce more superficial bites and muscle infection is likely not important in infection with bat RABV variants (Feder *et al.*, 1997).

13.3 RABV Receptors and Virus Entry

RABV enters the nervous system via a motor neuron through the neuromuscular junction (NMJ), or a sensory nerve through nerve spindles (Murphy *et al.*, 1973). The viral G protein is responsible for the attachment of the virus to target cells (Lafon, 2005). In 1982, the first identified receptor for RABV entry was nicotinic acetylcholine receptor (nAChR) by Lentz and coworkers, who showed that RABV bound to mouse diaphragms at the location of nAChR shortly after the immersion of mouse diaphragms in a suspension of RABV (Lentz *et al.*, 1986; Lentz, 1990). RABV antigen was found to co-localize with the nAChR in infected cultured myotubes from chicken embryos. It is evident from these studies that the distribution of viral antigen at the NMJ corresponds to the distribution of nAChRs. Pre-treatment of myotubes with either the irreversible binding nicotinic cholinergic antagonist α-bungarotoxin or the reversible binding *d*-tubocurarine reduced virus infection. Also, Tsiang *et al.* (1986) showed the inhibitory effect on RABV infection on rat myotubes *in vitro* by the pre-treatment of α-bungarotoxin. These research insights provide enough evidence that the RABV virus binds to nAChRs at the NMJ. Also, it has been established that the viral G protein is involved in attachment and entry into the target cells. Further investigation of RABV G protein binding to the nAChR suggests that both RABV and neurotoxins bind to residues 173–204 of the α_1-subunit of the nAChR. Also, the highest-affinity virus-binding residues of the α_1-subunit of the nAChR have been mapped to 179–192 (Lentz, 1990). In order to map the viral determinants for attachment and entry, Bracci *et al.* (1988) raised monoclonal antibodies against a peptide containing residues 190–203 of the RABV G protein. The monoclonal antibody inhibited the binding of the RABV G protein and α-bungarotoxin to the nAChR. These studies have provided strong evidence that RABV binds to nAChRs at the NMJ. Finally, Lentz *et al.* (1982) showed that the binding of RABV to AChRs, which is present in high concentrations at the NMJ, would localize and concentrate the virus and facilitate subsequent uptake and transfer of virus to peripheral motor nerves.

In addition to nAChR, other molecules have been proposed as RABV receptors. Thoulouze *et al.* (1998) reported that RABV may also bind to the neural cell adhesion molecule (NCAM), which is present in presynaptic membranes (Lafon, 2005). Incubation of susceptible cells with RABV decreased surface expression of NCAM. RABV infection could be inhibited when the NCAM receptor was blocked with heparan sulfate and by polyclonal or monoclonal antibodies directed against the NCAM receptor (Thoulouze *et al.*, 1998). Soluble NCAM can also neutralize RABV infection. In addition, significantly less RABV antigen was found in various brain regions in NCAM receptor-deficient than in wildtype mice, indicating that viral spread was less efficient without the NCAM receptor. However, lack of NCAM receptor *in vivo* only mildly delayed the death of mice. Since the NCAM receptor is localized in presynaptic membranes, it is well-positioned for RABV internalization

by receptor-mediated endocytosis into vesicles (Lafon, 2005). The low-affinity p75 neurotrophin receptor (p75NTR) has also been suggested to be an RABV receptor (Tuffereau *et al.*, 1998). Most cell lines of non-neuronal cell origin, including BSR cells, are not permissive for street RABV infection. However, BSR cells with stable expression of p75NTR were able to bind soluble RABV G protein. When p75NTR-deficient mice were infected intracerebrally with challenge virus standard, similar clinical features of disease and pathologic changes were observed in the brain as in mice expressing p75NTR (Jackson and Park, 1999). Tuffereau *et al.* (1998) performed further studies in cultured adult mouse dorsal root ganglion neurons and reported that, although p75NTR is a receptor for soluble RABV G protein in transfected cells of heterologous systems, a RABV G protein interaction is not necessary for RABV infection of primary neurons (Tuffereau *et al.*, 2007). p75NTR is not present at the NMJ and it is mainly present in the dorsal horn of the spinal cord, suggesting that it could be involved in trafficking of RABV by a sensory pathway (Lafon, 2005). It may also play an important role in the retrograde transport of RABV by forming an RABV–p75NTR complex that is transported into the cell in endocytic compartments, possibly following caveolae transcytosis.

Once RABV binds to presumptive receptor(s), virus entry is mediated by endocytosis. Studies performed in chick spinal cord muscle co-cultures show the co-localization of CVS strain of RABV and AChR tracers at the NMJ, which provides evidence that the NMJ is the major site of virus entry into neurons (Lewis *et al.*, 2000). Subsequently, co-localization with endosome tracers indicated that the virus resides in an early endosome compartment. There is also supporting ultrastructural evidence that RABV particles enter nerve terminals by endocytosis (Iwasaki and Clark, 1975; Charlton *et al.*, 1996). The acidic environment of the endosome triggers fusion of the viral membrane with the endosome membrane, which allows the viral nucleocapsid to escape into the cytoplasm. However, it has not yet been resolved whether the viral uncoating actually takes place in nerve terminals or in the cell body (perikaryon) after transport in the axon.

Although RABV infection with fixed strains is restricted to a small number of cell types *in vivo*, fixed viruses can infect a much larger variety of cell types *in vitro* (Reagan and Wunner, 1985). There is evidence that carbohydrate moieties, phospholipids, highly sialylated gangliosides and other membrane-associated proteins might contribute to the cellular membrane receptor structure for RABV (Superti *et al.*, 1984; Conti *et al.*, 1986).

13.4 RABV Spread

Once RABV enters the nervous system, it is transported in the fibres by centripetal spread within motor and perhaps also sensory axons of peripheral nerves. Colchicine, a microtubule-disrupting agent active for tubulin-containing cytoskeletal structures, is an effective inhibitor of fast axonal transport in the sciatic nerve of rats (Tsiang, 1979). When colchicine was applied locally to the sciatic nerve, propagation of RABV was prevented, which provides strong evidence that RABV spreads from sites of peripheral inoculation to the CNS by retrograde fast axonal transport. Using human dorsal root ganglia neurons Tsiang *et al.* (1991) showed that viral retrograde transport occurs at a rate of between 50 and 100 mm/day. There is evidence that the RABV P protein interacts with dynein light chain 8 (LC8) (Jacob *et al.*, 2000; Raux *et al.*, 2000). This led to speculation that RABV P protein–dynein interaction may be of fundamental importance in axonal transport of RABV. However, deletions of the dynein light chain-binding region of recombinant RABV demonstrated only minor effects on viral spread after peripheral inoculation (Mebatsion, 2001; Rasalingam *et al.*, 2005). Mazarakis *et al.* (2001) have demonstrated that RABV G protein-pseudotyped lentivirus (equine infectious anaemia virus)-based vectors enhance gene transfer to neurons

by facilitating retrograde axonal transport. Hence, RABV G protein may play a more important role than the P protein (Mazarakis *et al.*, 2001).

Viral spread within the CNS has been studied extensively in animal models. Once the virus invades the CNS neurons (often in the spinal cord) in rodent models there is rapid dissemination of RABV along neuroanatomical pathways by fast axonal transport. Studies using stereotaxic brain inoculation in rats show the axonal transport of virus in the neuroanatomical structures (Gillet *et al.*, 1986) and it is supported by studies in which the administration of colchicine inhibited virus transport within the CNS (Ceccaldi *et al.*, 1989, 1990). Tsiang *et al.* (1989) showed the anterograde fast axonal movement of RABV is in the range of 100–400 mm/day in cultured rat dorsal root ganglia neurons. However, it is contradictory to studies performed in rhesus monkeys, which indicate that the spread of RABV occurs exclusively by retrograde axonal transport (Kelly and Strick, 2000). Studies performed with G-deficient recombinant RABV showed limited spread in the brains of mice after intracerebral inoculation (Etessami *et al.*, 2000). Therefore, it is clear that the G protein is necessary for trans-synaptic spread of RABV from one neuron to another. In a skunk model, it has been found that most viral budding occurs on synaptic or adjacent plasma membranes of dendrites, with less prominent budding from the plasma membrane of the perikaryon and occasionally budding freely into the intracellular space (Charlton and Casey, 1979). Also, the presence of virus partially engulfed in the invaginated membrane of an adjacent axon terminal indicates transneuronal dendroaxonal transfer of virus.

Experiments involving the inoculation of CVS in the footpad of mice shows there was early involvement of neurons in the brainstem tegmentum and deep cerebellar nuclei (Jackson and Reimer, 1989). Subsequently, the virus spreads to involve cerebellar Purkinje cells and neurons in the diencephalon, basal ganglia and cerebral cortex. RABV spreads to the hippocampus relatively late after peripheral inoculation (Carbone *et al.*, 1987). RABV predominantly infects pyramidal neurons of the hippocampus, with relative sparing of neurons in the dentate gyrus in adult mice (Jackson and Reimer, 1989). Although the basis of specific cell tropism is still unclear, Gosztonyi and Ludwig (2001) speculated that the cell selectivity can be explained if *N*-methyl-D-aspartate (NMDA) NR1 receptors are involved as RABV receptors, because RABV spreads only by retrograde (not by anterograde) fast axonal transport. Therefore, the virus cannot infect dentate granule cells by the perforant path and mossy fibres from CA3 that predominantly have α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and kainate receptors rather than NMDA receptors. In experimentally infected skunks, it has been observed that skunk RABV is highly neurotropic and infects Bergmann glia in the cerebellum more prominently than Purkinje cells (Jackson *et al.*, 2000). Initial infection was present in the lumbar spinal cord and transit to the brain occurred via a variety of long ascending and descending fibre tracts, including rubrospinal, corticospinal, spinothalamic, spino-olivary, vestibulospinal/spinovestibular, reticulospinal/spinoreticular, cerebellospinal/spinocerebellar and dorsal column pathways (Charlton *et al.*, 1996).

13.5 RABV as an Advanced Tracer for Neural Circuits

RABV acts as a highly effective trans-neuronal tracer (Ugolini, 1995, 2008; Kelly and Strick, 2000; Callaway, 2008; Lyon, 2012). As noted above, the viral G protein serves as the ligand necessary for infection and transneuronal spread (Conzelmann *et al.*, 1990; Gaudin *et al.*, 1993; Etessami *et al.*, 2000). And, because the G protein receptor appears to be localized to presynaptic nerve terminals (Lafon, 2005), injections of RABV directly into the brain infect neurons through axon terminals and RABV is transported in a retrograde direction to replicate in the soma, then invade postsynaptic terminals in order to spread retrogradely

through additional neurons (Kelly and Strick, 2000; Callaway, 2008; Ugolini, 2008; Lyon, 2012).

Unlike traditional non-viral retrograde neuronal tracers, neurons are very well labelled by RABV, allowing for detailed morphological reconstruction following immunohistochemical staining for the RABV N protein (Nassi *et al.*, 2006). However, in order to extract meaningful data, the spread of the virus needs to be prevented from infecting the entire CNS. One approach for controlling RABV spread is to limit the survival time after injection. The CVS strain, for example, can be limited to two synapses using a survival time of 72 hours (Callaway, 2008). This approach has been used to identify several previously unknown di-synaptic pathways in monkeys (Nassi *et al.*, 2006; Nassi and Callaway, 2009; Lyon *et al.*, 2010; Lyon and Rabideau, 2012). Therefore, controlled use of fixed strains of RABV can be an effective tool.

The spread of RABV can also be restricted by altering the genetic expression of the G protein (Etessami *et al.*, 2000). Wickersham and colleagues (2007a) removed the G gene from the SAD-B19 vaccine strain and replaced it with the gene for enhanced GFP (Fig. 13.2). Moreover, during production of this G-deleted (ΔG) RABV (SADΔG-GFP), the G protein was supplied so that it could be incorporated into the viral envelope (but not into the genome). Thus, the virus acts as a monosynaptic retrograde tracer, infecting axon terminals at the site of brain injection, transporting retrogradely to the soma where it replicates and fills the soma and dendrites completely with GFP (Nassi and Callaway, 2007; Wickersham *et al.*, 2007a; Connolly *et al.*, 2012), but is unable to spread to presynaptically connected neurons because the G gene is missing (Fig. 13.2B). Variants of this virus are now used to deliver many different genes, from red or blue fluorescent proteins, to genetically encoded Ca^{2+} indicators for imaging cell activity, to channelrhodopsin-2 for light activation, and many others (Osakada *et al.*, 2011).

An additional modification to the SADΔG-GFP was made to enable highly specific, genetically targeted infection of a single neuron. Wickersham and colleagues (2007a) replaced the G protein in the membrane envelope with the envelope A protein (EnvA) from the avian sarcoma and leukosis virus, a process known as pseudotyping (Fig. 13.2A). EnvA limits infection to cells that express the TVA receptor, a protein found in birds but not mammals (Barnard *et al.*, 2006). As developed by Wickersham *et al.* (2007b) for the resulting pseudotyped virus, EnvA-SADΔG-GFP, to work as a 'single-cell tracer' three genes must be delivered to the target neuron prior to delivering the virus (Fig. 13.2C–F): (1) the gene for TVA, so that the virus can enter the soma; (2) the gene for RABV G protein, to allow the virus spread to presynaptically connected neurons; and (3) the gene for a marker protein such as mCherry for cell identification. Following a 24-hour period to allow for expression of these three proteins, the EnvA-SADΔG-GFP virus is injected near the transfected neuron. The transfected neuron, which is already expressing mCherry (Fig. 13.2E), will become infected and then over the next day begin to express GFP (Fig. 13.2F); after a few additional days presynaptically connected neurons from all over the brain will also become infected and express GFP as well (Fig. 13.2D, F). The virus will not spread beyond these directly connected presynaptic cells since the gene for RABV G protein was not delivered to these cells. *In vivo*, this technique can label inputs originating throughout the brain providing unprecedented global detail of the circuits involving a single neuron (Marshel *et al.*, 2010; Rancz *et al.*, 2011).

13.6 Spread from the CNS

The viral centrifugal spread from the CNS to peripheral sites including salivary glands along neuronal routes is essential for efficient transmission of RABV among its natural hosts. Anatomically, salivary glands receive parasympathetic innervation by the facial (via the submandibular ganglion or Langley's ganglion in some animals) and

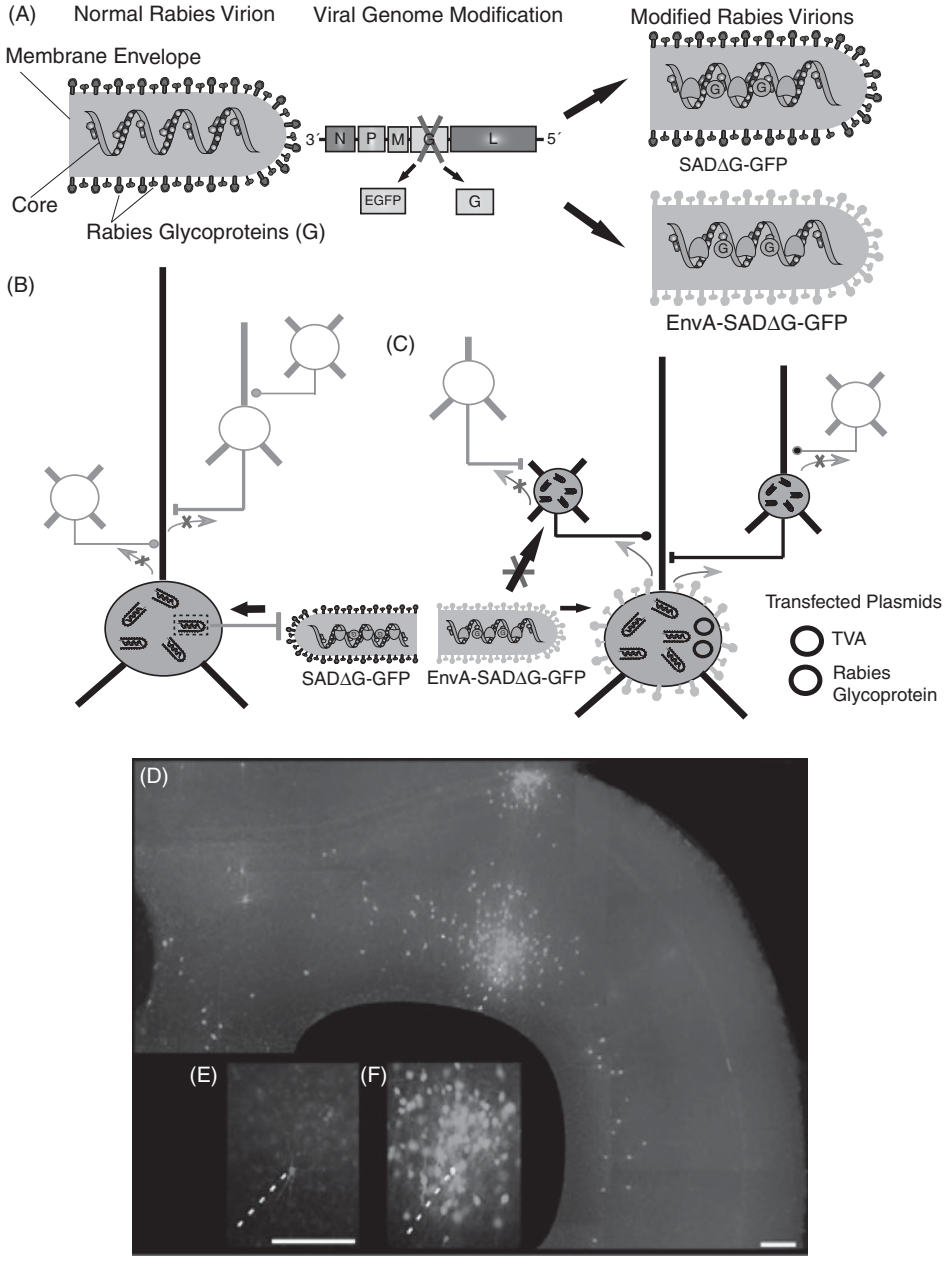

Fig. 13.2. Using a modified and pseudotyped rabies virus to retrogradely trace the inputs to a single neuron. (A) Schematic of the rabies virion. (B) Retrograde infection with SADΔG-GFP. The SADΔG-GFP virus acts as a non-specific retrograde tracer, infecting all terminal endings at the injection site. (C, D) Targeting infection of a single neuron and labelling of local and long-range presynaptic neurons with EnvA-SADΔG-GFP. (E) GFP expressing cells 24 hours post infection. (F) Over a few more days presynaptic neurons will also become infected and begin to express GFP as well. The virus cannot spread beyond these directly connected presynaptic cells because no rabies glycoprotein gene is present. Panels A and C–F are modified from Wickersham *et al.* (2007b). Scale bar: 200μm.

glossopharyngeal (via the optic ganglion) nerves, sympathetic innervation via the superior (or cranial) cervical ganglion and afferent (sensory) innervation (Emmelin, 1967). Experimental studies using street RABV in dogs and foxes involving unilateral excision of a portion of the lingual nerve and the cranial cervical ganglion resulted in very low viral titres in denervated salivary glands compared with contralateral salivary glands (Dean *et al.*, 1963). Also, it is evident from the studies by Charlton *et al.* (1983) that the widespread infection of salivary gland epithelial cells is a result of viral spread along multiple terminal axons rather than spread between epithelial cells. Experimental rabies infection of non-nervous tissues in skunks and foxes by Balachandran and Charlton (1994) shows the presence of RABV antigen in the apical region of mucous acinar cells. Also, the ultrastructural studies show the presence of viral proteins in tissues at variable proportion, and also suggested that the viral titres in salivary glands may be higher than in CNS tissues.

In addition to salivary gland infection, there is mounting evidence in experimental animal models that the centrifugal spread of virus involves distal sites in the central, peripheral and autonomic nervous systems, including corneal epithelial cells and the ganglion cell layer of the retina (Murphy *et al.*, 1973; Balachandran and Charlton, 1994). It is important to note that RABV has been transmitted by corneal transplantation in humans in eight well-documented cases (Jackson, 2002). Detection of RABV antigen in corneal impression smears has been used as a diagnostic test for human rabies (Koch *et al.*, 1975). In addition, infection may be found in free sensory nerve endings of tactile hair in a skin biopsy, which is one of the best diagnostic methods of confirming an ante-mortem diagnosis of rabies in humans.

Studies in both natural and experimental rabies have demonstrated infection involving neurons and non-neuronal cells. RABV infection spread is demonstrated in a variety of extraneural organs, including the adrenal medulla, cardiac ganglia and plexuses in the luminal gastrointestinal tract, major salivary glands, liver and exocrine pancreas (Debbie and Trimarchi, 1970; Balachandran and Charlton, 1994; Jackson *et al.*, 1999). In addition, it is also shown that RABV infects non-neuronal cells, including acini in major salivary glands in rabies vectors, epithelium of the tongue, cardiac and skeletal muscle, hair follicles, and even pancreatic islets (Debbie and Trimarchi, 1970; Murphy *et al.*, 1973; Balachandran and Charlton, 1994; Jackson *et al.*, 1999). Moreover, there are a few reports of myocarditis in human cases of rabies (Ross and Armentrout, 1962; Cheetham *et al.*, 1970).

13.7 Molecular Diagnosis

Rabies is a zoonotic viral infectious disease transmitted from animals to humans. Rabies is widely distributed across the globe, with only a few countries (mainly islands and peninsulas) being free of the disease. Dogs, raccoons, skunks, bats and foxes are important rabies vectors. A person or animal can contract the disease in many ways; most commonly by a bite from an already infected animal. Non-bite exposures and human to human transmission are rare (Yousaf *et al.*, 2012). Rabies is caused by the virus that infects the CNS with initial flu-like symptoms, including fever and headache, and the infection progresses quickly to paralysis.

Rapid and accurate laboratory diagnosis of rabies in humans and other animals is essential for timely administration of post-exposure prophylaxis. Rapid laboratory diagnosis may result in avoiding unnecessary prophylactic therapy, which is associated with emotional stress and potential adverse effects as well as a financial burden if the animal is not rabid (Centers for Disease Control and Prevention guidelines: Manning *et al.*, 2008). In addition, laboratory identification of positive rabies cases may aid in defining current epidemiologic patterns of disease and provide appropriate information for the development of rabies control programmes.

Historically, histopathological techniques such as the Sellers staining technique were

used to detect the presence of Negri bodies. However, this technique is no longer recommended by WHO (Whorton *et al.*, 1993) due to poor sensitivity and specificity. Microscopic analysis of brain tissue samples for RABV-specific antigen is the only direct method that allows diagnostic confirmation of rabies in a short time and at a reduced cost, irrespective of geographical origin and status of the host. It has to be regarded as the first step in diagnostic procedures for all laboratories. The fluorescent antibody test (FAT) (Serfaty-Lacrosniere *et al.*, 1994) relies on the ability of a detector molecule (fluorescein isothiocyanate) coupled with a RABV-specific antibody forming a conjugate to bind to and allow the visualization of RABV antigen using fluorescent microscopy techniques. However, the autolysed samples can reduce the sensitivity and specificity of the FAT. In addition, FAT requires fluorescent microscopes that are not always available, particularly in developing countries. Recently, the Centers for Disease Control and Prevention (CDC) developed the direct rapid immunohistochemistry test (dRIT) (Lembo *et al.*, 2006), which is similar to the dFA. Brain smears or imprints on glass slides are fixed with 10% buffered formalin. According to standard immunohistochemical staining, the virus antigen can be detected by anti-RABV N monoclonal antibody and examined under a light microscope. The sensitivity and specificity of the dRIT are equivalent to those of the dFA without the need for a fluorescent microscope.

The rabies tissue culture infection test (RTCIT) (Sureau, 1986) and the mouse inoculation test (MIT) (Webster *et al.*, 1976) were used to detect virus particles indirectly based on the propagation and isolation of the virus in tissue culture and in animals, respectively. However, the conventional diagnostic tests were unreliable. These tests are rarely optimal and are entirely dependent on the nature and the quality of the samples. In the course of the past three decades, the application of molecular biology techniques has aided in the development of tests that were more rapid and highly sensitive for the detection of RABV. The advent of molecular biology has changed the face of diagnostic virology, generally enabling short turnaround-times and high throughput. These molecular techniques not only enable us to rapidly detect the presence of virus but also enable us to detect the virus strain from clinical samples. Currently, there are a number of molecular tests that can be used to complement conventional tests in rabies diagnosis.

13.7.1 Nucleic acid detection-based assays

Reverse-transcriptase polymerase chain reaction (RT-PCR)

The polymerase chain reaction (PCR)-based platform technologies have, in particular, quickly revolutionized infectious disease diagnoses (Tarr and Frei, 2010). The nucleic acid amplification platforms are sensitive, specific, rapid and robust, therefore ideal for high throughput. To detect RNA viruses, a cDNA copy of the RNA must first be made using reverse transcriptase (RT). The cDNA then acts as the template for amplification by the PCR. This technique is referred to as reverse transcription PCR (RT-PCR). Since the primers were selected from the conserved regions of the genome, most assays amplify parts of the N gene. Various conventional RT-PCR protocols for the diagnostic amplification of lyssavirus genome fragments have been published (Fooks *et al.*, 2009). The molecular approaches intended to detect all lyssaviruses have applications for both ante-mortem (saliva, cerebrospinal fluid, brain) and post-mortem samples (Fooks *et al.*, 2009). These molecular diagnostic procedures are also applied for further virus characterization including sequencing reactions or restriction fragment length polymorphism (RFLP) (Johnson *et al.*, 2002). Also, RT-PCRs have been modified by using strain-specific primers to distinguish various RABV strains. Thus, the classical RT-PCR assays proved to be a sensitive and specific tool for routine diagnostic purposes, particularly in decomposed samples or archival specimens. However, the sole detection of

the amplified RT-PCR products does not allow an exact quantification of genome copies.

TaqMan RT-PCR

Various conventional, gel-based PCR assays for the amplification of RNA fragments are available. However, these techniques do not allow the exact quantification of viral genome copies. To overcome this, several methods have been developed for rabies diagnosis, including hybridization, restriction fragment length polymorphism (RFLP), PCR-enzyme-linked immunosorbent assay *in situ* hybridization, and sequencing.

TaqMan RT-PCR uses flurogenic probes for the detection of sequence-specific templates in real time. Wakeley *et al.* (2005) developed an assay using generic and genotype-specific probes to detect and differentiate lyssavirus genotypes. Later this assay was improvised for the rapid detection of amplicons using specific dye such as SYBR green. However, a single mutation of the primers or the probe can alter the sensitivity of the PCR (Hughes *et al.*, 2004). Thus, given the genetic diversity of the lyssavirus variants, one has to be careful when relying on one single assay for detection and differentiation.

Nucleic acid sequence-based amplification (NASBA)

NASBA is a single-step isothermal amplification technology that uses three enzymes, namely reverse transcriptase, RNase H and T7 RNA polymerase to synthesize multiple copies of target RNA under isothermal conditions. Briefly, a pair of specific primers was used to generate a large number of RNA copies, of which one primer contains the T7 RNA polymerase binding site and the other contains the electrochemiluminescence detection region attached to the 5′ end. Amplified RNA products are detected by hybridization using electrochemiluminescence (Leclercq *et al.*, 2011) labelled probes by an automated reader (Wacharapluesadee and Hemachudha, 2001). It is relatively easy to use and the processing to detection time is 4 hours. The NASBA technique has also been widely used to investigate various bacterial and viral pathogens (Zaytseva *et al.*, 2004).

Loop-mediated isothermal amplification (LAMP)

The LAMP assay was first described by Notomi *et al.* (2000; Mori and Notomi, 2009). LAMP offers an alternative DNA amplification method to the polymerase chain reaction for amplification of nucleic acids with high specificity, without the need of sophisticated instruments like thermal cyclers. Briefly, the principle of LAMP is based on strand displacement by *Bst* polymerase and formation of the stem-loop structure by the four specifically designed primers that can recognize six distinct regions on the target DNA. This reaction is conducted under isothermal conditions using conventional water bath or heat blocks. Thus, this method is ideal for diagnosing rabies in both humans and animals in developed and underdeveloped countries. A reverse transcription step is undertaken prior to the LAMP for the amplification of RNA viruses. This technique generates a large amount of target sequences within minutes. Primer sets have been successfully developed to detect a range of pathogenic viruses, including RABV, West Nile virus (Parida *et al.*, 2004), Japanese encephalitis virus (Toriniwa and Komiya, 2006), foot and mouth disease virus (Dukes *et al.*, 2006) and Chikungunya virus (Parida *et al.*, 2007). However, sequence variation in RABV genome poses a major challenge in the use of RT-LAMP assay for use in diagnosis and surveillance.

Microarray detection of lyssaviruses

Microarray technology has become a rapid and efficient method in clinical diagnostics (Wang *et al.*, 2002; Boonham *et al.*, 2007). Microarray-based detection chips have been reported for the detection and genotyping of variety of viruses including influenza virus, hepatitis B virus, foot and mouth disease virus and respiratory viruses (Jack *et al.*, 2009). Recently, Gurrala *et al.* (2009) developed a DNA microarray for detection and

differentiation of lyssaviruses. Briefly, lyssavirus RNA was converted to cDNA and amplified in a random PCR, labelled with fluorescent dye and hybridized to probes on the microarray chip for detection. The N gene was selected as the target because it is conserved among the lyssavirus genotypes. These high throughput molecular techniques not only enable us to rapidly detect the presence of various viruses but also enable us to detect and differentiate the virus strain from the clinical samples.

13.8 Conclusions

Rabies is a zoonotic disease that remains an important public health problem worldwide and causes more than 55,000 human deaths every year. Rabies virus is a negative-stranded RNA virus of the *Rhabdoviridae* family, which is the causative agent of rabies. Rabies virus is often transmitted through the bite of a rabid animal. Rabies virus infects the CNS of the host; neuroinvasiveness and neurotropism are the main features that define the pathogenesis of rabies. Considerable progress has been made in understanding the pathogenesis of rabies. At the bite site, rabies virus binds to the nAChR at the NMJ and it spreads by retrograde axonal transport via peripheral nerves to the CNS, from the CNS virus spreads centrifugally to various organs including salivary glands to allow successive efficient transmission of the infection. Recent research in a variety of animal models using virus variants indicates the essential role of rabies virus glycoprotein (G protein) on neurotropism and rapid axonal/transsynaptic spread of rabies virus, thereby on neurovirulence. In spite of much effort, the precise events at the site of viral entry during the long incubation period and neuronal dysfunction are unclear. A better understanding of rabies spread, neuronal dysfunction and immune evasion will hopefully lead to advances in the treatment of rabies and other viral diseases.

References

Baer, G.M. and Cleary, W.F. (1972) A model in mice for the pathogenesis and treatment of rabies. *Journal of Infectious Diseases* 125, 520–527.

Baer, G.M., Shantha, T.R. and Bourne, G.H. (1968) The pathogenesis of street rabies virus in rats. *Bulletin of the World Health Organization* 38, 119–125.

Balachandran, A. and Charlton, K. (1994) Experimental rabies infection of non-nervous tissues in skunks (*Mephitis mephitis*) and foxes (*Vulpes vulpes*). *Veterinary Pathology* 31, 93–102.

Barnard, R.J., Elleder, D. and Young, J.A. (2006) Avian sarcoma and leukosis virus-receptor interactions: from classical genetics to novel insights into virus-cell membrane fusion. *Virology* 334, 25–29.

Boonham, N., Tomlinson, J. and Mumford, R. (2007) Microarrays for rapid identification of plant viruses. *Annual Review of Phytopathology* 45, 307–328.

Bracci, L., Antoni, G., Cusi, M.G., Lozzi, L., Niccolai, N., Petreni, S., Rustici, M., Santucci, A., Soldani, P. and Valensin, P.E. (1988) Antipeptide monoclonal antibodies inhibit the binding of rabies virus glycoprotein and alpha-bungarotoxin to the nicotinic acetylcholine receptor. *Molecular Immunology* 25, 881–888.

Callaway, E.M. (2008) Transneuronal circuit tracing with neurotropic viruses. *Current Opinion in Neurobiology* 18, 617–623.

Carbone, K.M., Duchala, C.S., Griffin, J.W., Kincaid, A.L. and Narayan, O. (1987) Pathogenesis of borna disease in rats – evidence that intraaxonal spread is the major route for virus dissemination and the determinant for disease incubation. *Journal of Virology* 61, 3431–3440.

Ceccaldi, P.E., Gillet, J.P. and Tsiang, H. (1989) Inhibition of the transport of rabies virus in the central nervous-system. *Journal of Neuropathology and Experimental Neurology* 48, 620–630.

Ceccaldi, P.E., Ermine, A. and Tsiang, H. (1990) Continuous delivery of colchicine in the rat-brain with osmotic pumps for inhibition of rabies virus transport. *Journal of Virological Methods* 28, 79–84.

Charlton, K.M. and Casey, G.A. (1979) Experimental rabies in skunks – immunofluorescence light and electron-microscopic studies. *Laboratory Investigation* 41, 36–44.

Charlton, K.M., Casey, G.A. and Campbell, J.B. (1983) Experimental rabies in skunks – mechanisms of infection of the salivary-glands. *Canadian Journal of Comparative Medicine – Revue Canadienne de Médecine Comparee* 47, 363–369.

Charlton, K.M., Casey, G.A., Wandeler, A.I. and Nadin-Davis, S. (1996) Early events in rabies virus infection of the central nervous system in skunks (*Mephitis mephitis*). *Acta Neuropathologica* 91, 89–98.

Charlton, K.M., Nadin-Davis, S., Casey, G.A. and Wandeler, A.I. (1997) The long incubation period in rabies: delayed progression of infection in muscle at the site of exposure. *Acta Neuropathologica* 94, 73–77.

Cheetham, H.D., Hart, J., Coghill, N.F. and Fox, B. (1970) Rabies with myocarditis – two cases in England. *The Lancet* 295, 921–922.

Connolly, J.D., Hashemi-Nezhad, M. and Lyon, D.C. (2012) Parallel feedback pathways in visual cortex of cats revealed through a modified rabies virus. *Journal of Comparative Neurology* 520, 988–1004.

Conti, C., Superti, F. and Tsiang, H. (1986) Membrane carbohydrate requirement for rabies virus binding to chicken embryo related cells. *Intervirology* 26(3), 164–168.

Conzelmann, K.K., Cox, J.H., Schneider, L.G. and Thiel, H.J. (1990) Molecular cloning and complete nucleotide sequence of the attenuated rabies virus SAD B19. *Virology* 175(2), 485–499.

Dean, D.J., Evans, W.M. and Mcclure, R.C. (1963) Pathogenesis of rabies. *Bulletin of the World Health Organization* 29, 803–811.

Debbie, J.G. and Trimarchi, C.V. (1970) Pantropism of rabies virus in free-ranging rabid red fox *Vulpes fulva*. *Journal of Wildlife Diseases* 6, 500–506.

Dierks, R.E. (1979) Rabies pathogenesis and diagnosis. *Journal of Laboratory and Clinical Medicine* 94, 1–4.

Dietzschold, B., Schnell, M. and Koprowski, H. (2005) Pathogenesis of rabies. *Current Topics in Microbiology and Immunology* 292, 45–56.

Dietzschold, B., Li, J., Faber, M. and Schnell, M. (2008) Concepts in the pathogenesis of rabies. *Future Virology* 3, 481–490.

Dukes, J.P., King, D.P. and Alexandersen, S. (2006) Novel reverse transcription loop-mediated isothermal amplification for rapid detection of foot-and-mouth disease virus. *Archives of Virology* 151, 1093–1106.

Emmelin, N. (1967) Parotid secretion after cutting auriculotemporal nerve at different levels. *Journal of Physiology* 188, 44.

Etessami, R., Conzelmann, K.K., Fadai-Ghotbi, B., Natelson, B., Tsiang, H. and Ceccaldi, P.E. (2000) Spread and pathogenic characteristics of a G-deficient rabies virus recombinant: an in vitro and in vivo study. *Journal of General Virology* 81, 2147–2153.

Feder, H.M. Jr, Nelson, R. and Reiher, H.W. (1997) Bat bite? *The Lancet* 350, 1300.

Fooks, A.R., Johnson, N., Freuling, C.M., Wakeley, P.R., Banyard, A.C., McElhinney, L.M., Marston, D.A., Dastjerdi, A., Wright, E., Weiss, R.A. and Müller, T. (2009) Emerging technologies for the detection of rabies virus: challenges and hopes in the 21st century. *PLoS Neglected and Tropical Diseases* 3, e530.

Fu, Z.F. (1997) Rabies and rabies research: past, present and future. *Vaccine* 15, S20–24.

Gaudin, Y., Ruigrok, R.W., Knossow, M. and Flamand, A. (1993) Low-pH conformational changes of rabies virus glycoprotein and their role in membrane fusion. *Journal of Virology* 67, 1365–1372.

Gillet, J.P., Derer, P. and Tsiang, H. (1986) Axonal-transport of rabies virus in the central-nervous-system of the rat. *Journal of Neuropathology and Experimental Neurology* 45, 619–634.

Gosztonyi, G. and Ludwig, H. (2001) Interactions of viral proteins with neurotransmitter receptors may protect or destroy neurons. *Mechanisms of Neuronal Damage in Virus Infections of the Nervous System* 253, 121–144.

Gurrala, R., Dastjerdi, A., Johnson, N., Nunez-Garcia, J., Grierson, S., Steinbach, F. and Banks, M. (2009) Development of a DNA microarray for simultaneous detection and genotyping of lyssaviruses. *Virus Research* 144, 202–208.

Hooper, D.C. (2005) The role of immune responses in the pathogenesis of rabies. *Journal of Neurovirology* 11, 88–92.

Hughes, G.J., Smith, J.S., Hanlon, C.A. and Rupprecht, C.E. (2004) Evaluation of a TaqMan PCR assay to detect rabies virus RNA: influence of sequence variation and application to quantification of viral loads. *Journal of Clinical Microbiology* 42, 299–306.

Iwasaki, Y. and Clark, H.F. (1975) Cell to cell transmission of virus in the central nervous system. II. Experimental rabies in mouse. *Laboratory Investigations* 33, 391–399.

Jack, P.J., Amos-Ritchie, R.N., Reverter, A., Palacios, G., Quan, P.L., Jabado, O., Briese, T., Lipkin, W.I. and Boyle, D.B. (2009) Microarray-based detection of viruses causing vesicular or vesicular-like lesions in livestock animals. *Veterinary Microbiology* 133, 145–153.

Jackson, A.C. (2002) Rabies pathogenesis. *Journal of Neurovirology* 8, 267–269.

Jackson, A.C. (2006) Rabies: new insights into pathogenesis and treatment. *Current Opinions in Neurology* 19, 267–270.

Jackson, A.C. and Park, H. (1999) Experimental rabies virus infection of p75 neurotrophin receptor-deficient mice. *Acta Neuropathologica* 98, 641–644.

Jackson, A.C. and Reimer, D.L. (1989) Pathogenesis of experimental rabies in mice – an immunohistochemical study. *Acta Neuropathologica* 78, 159–165.

Jackson, A.C., Ye, H., Phelan, C.C., Ridaura-Sanz, C., Zheng, Q., Li, Z., Wan, X. and Lopez-Corella, E. (1999) Extraneural organ involvement in human rabies. *Laboratory Investigation* 79, 945–951.

Jackson, A.C., Phelan, C.C. and Rossiter, J.P. (2000) Infection of Bergmann glia in the cerebellum of a skunk experimentally infected with street rabies virus. *Canadian Journal of Veterinary Research – Revue Canadienne de Recherche Vétérinaire* 64, 226–228.

Jacob, Y., Badrane, H., Ceccaldi, P.E. and Tordo, N. (2000) Cytoplasmic dynein LC8 interacts with lyssavirus phosphoprotein. *Journal of Virology* 74, 10,217–10,222.

Johnson, N., McElhinney, L.M., Smith, J., Lowings, P. and Fooks, A.R. (2002) Phylogenetic comparison of the genus Lyssavirus using distal coding sequences of the glycoprotein and nucleoprotein genes. *Archives of Virology* 147, 2111–2123.

Kelly, R.M. and Strick, P.L. (2000) Rabies as a transneuronal tracer of circuits in the central nervous system. *Journal of Neuroscience Methods* 103, 63–71.

Koch, F.J., Sagartz, J.W., Davidson, D.E. and Lawhaswasdi, K. (1975) Diagnosis of human rabies by cornea test. *American Journal of Clinical Pathology* 63, 509–515.

Lafon, M. (2005) Rabies virus receptors. *Journal of Neurovirology* 11, 82–87.

Lawson, D.M., Artymiuk, P.J., Yewdall, S.J., Smith, J.M., Livingstone, J.C., Treffry, A., Luzzago, A., Levi, S., Arosio, P., Cesareni, G., Thomas, C.D., Shaw, W.V. and Harrison, P.M. (1991) Solving the structure of human H ferritin by genetically engineering intermolecular crystal contacts. *Nature* 349, 541–544.

Leclercq, S.Y., dos Santos, R.M., Macedo, L.B., Campos, P.C., Ferreira, T.C., de Almeida, J.G., Seniuk, J.G., Serakides, R., Silva-Cunha, A. and Fialho, S.L. (2011) Evaluation of water-in-oil-in-water multiple emulsion and microemulsion as potential adjuvants for immunization with rabies antigen. *European Journal of Pharmaceutical Sciences* 43, 378–385.

Lembo, T., Niezgoda, M., Velasco-Villa, A., Cleaveland, S., Ernest, E. and Rupprecht, C.E. (2006) Evaluation of a direct, rapid immunohistochemical test for rabies diagnosis. *Emerging Infectious Diseases* 12, 310–313.

Lenard, J. (1996) Negative-strand virus M and retrovirus MA proteins: all in a family? *Virology* 216, 289–298.

Lentz, T.L. (1990) Rabies virus binding to an acetylcholine receptor alpha-subunit peptide. *Journal of Molecular Recognition* 3, 82–88.

Lentz, T.L., Burrage, T.G., Smith, A.L., Crick, J. and Tignor, G.H. (1982) Is the acetylcholine-receptor a rabies virus receptor. *Science* 215, 182–184.

Lentz, T.L., Benson, R.J., Klimowicz, D., Wilson, P.T. and Hawrot, E. (1986) Binding of rabies virus to purified Torpedo acetylcholine receptor. *Brain Research* 387, 211–219.

Lewis, P., Fu, Y. and Lentz, T.L. (2000) Rabies virus entry at the neuromuscular junction in nerve-muscle cocultures. *Muscle Nerve* 23, 720–730.

Lyon, D.C. (2012) Morphological approaches to the anatomical dissection of neuronal circuits. In Fellin, T. and Halassa, M. (eds) *Neuronal Network Analysis: Concepts and Experimental Approaches*. Springer, New York. Vol. 67, pp. 375–389.

Lyon, D.C. and Rabideau, C. (2012) Lack of robust LGN label following transneuronal rabies virus injections into macaque area V4. *Journal of Comparative Neurology* 520, 2500–2511.

Lyon, D.C., Nassi, J.J. and Callaway, E.M. (2010) A disynaptic relay from superior colliculus to dorsal stream visual cortex in macaque monkey. *Neuron* 65, 270–279.

Manning, S.E., Rupprecht, C.E., Fishbein, D., Hanlon, C.A., Lumlertdacha, B., Guerra, M., Meltzer, M.I., Dhankhar, P., Vaidya, S.A., Jenkins, S.R., Sun, B. and Hull, H.F. (2008) Human rabies prevention – United States, 2008: recommendations of the Advisory Committee on Immunization Practices. *MMWR Recommendations and Reports* 57, 1–28.

Marshel, J.H., Garrett, M.E. Nauhaus, I. and Callaway, E.M. (2010) Functional specialization of seven mouse visual cortical areas. *Neuron* 72, 1040–1054.

Mazarakis, N.D., Azzouz, M., Rohll, J.B., Ellard, F.M., Wilkes, F.J., Olsen, A.L., Carter, E.E., Barber, R.D., Baban, D.F., Kingsman, S.M., Kingsman, A.J., O'Malley, K. and Mitrophanous, K.A. (2001) Rabies virus glycoprotein pseudotyping of lentiviral vectors enables retrograde axonal transport and access to the nervous system after peripheral delivery. *Human Molecular Genetics* 10, 2109–2121.

Mebatsion, T. (2001) Extensive attenuation of rabies virus by simultaneously modifying the dynein light chain binding site in the P protein and replacing Arg333 in the G protein. *Journal of Virology* 75, 11,496–11,502.

Mori, Y. and Notomi, T. (2009) Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. *Journal of Infections and Chemotherapy* 15, 62–69.

Morimoto, K., Hooper, D.C., Carbaugh, H., Fu, Z.F., Koprowski, H. and Dietzschold, B. (1998) Rabies virus quasispecies: implications for pathogenesis. *Proceedings of the National Academy of Sciences USA* 95, 3152–3156.

Murphy, F.A., Harrison, A.K., Winn, W.C. and Bauer, S.P. (1973) Comparative pathogenesis of rabies and rabies-like viruses. Viral infection and transit from inoculation site to the central nervous system. *Laboratory Investigation* 28, 361–376.

Murphy, F.A., Harrison, A.K., Winn, W.C. and Bauer, S.P. (1973) Comparative pathogenesis of rabies and rabies-like viruses: infection of the central nervous system and centrifugal spread of virus to peripheral tissues. *Laboratory Investigation* 29, 1–16.

Nassi, J.J. and Callaway, E.M. (2007) Specialized circuits from primary visual cortex to V2 and area MT. *Neuron* 55, 799–808.

Nassi, J.J. and Callaway, E.M. (2009) Parallel processing strategies of the primate visual system. *Nature Reviews in Neurosciences* 10, 360–372.

Nassi, J.J., Lyon, D.C. and Callaway, E.M. (2006) The parvocellular LGN provides a robust disynaptic input to the visual motion area MT. *Neuron* 50, 319–327.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. and Hase, T. (2000) Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research* 28, e63.

Osakada, F., Mori, T., Cetin, A.H., Marshel, J.H., Virgen, B. and Callaway, E.M. (2011) New rabies virus variants for monitoring and manipulating activity and gene expression in defined neural circuits. *Neuron* 71, 617–631.

Parida, M., Posadas, G., Inoue, S., Hasebe, F. and Morita, K. (2004) Real-time reverse transcription loop-mediated isothermal amplification for rapid detection of West Nile virus. *Journal of Clinical Microbiology* 42, 257–263.

Parida, M.M., Santhosh, S.R., Dash, P.K., Tripathi, N.K., Lakshmi, V., Mamidi, N., Shrivastva, A., Gupta, N., Saxena, P., Babu, J.P., Rao, P.V. and Morita, K. (2007) Rapid and real-time detection of Chikungunya virus by reverse transcription loop-mediated isothermal amplification assay. *Journal of Clinical Microbiology* 45, 351–357.

Rancz, E.A., Franks, K.M., Schwarz, M.K., Pichler, B., Schaefer, A.T. and Margrie, T.W. (2011) Transfection via whole-cell recording in vivo: bridging single-cell physiology, genetics and connectomics. *Nature Neurosciences* 14, 527–532.

Rasalingam, P., Rossiter, J.P., Mebatsion, T. and Jackson, A.C. (2005) Comparative pathogenesis of the SAD-L16 strain of rabies virus and a mutant modifying the dynein light chain binding site of the rabies virus phosphoprotein in young mice. *Virus Research* 111, 55–60.

Raux, H., Flamand, A. and Blondel, D. (2000) Interaction of the rabies virus P protein with the LC8 dynein light chain. *Journal of Virology* 74, 10,212–10,216.

Ray, N.B., Ewalt, L.C. and Lodmell, D.L. (1995) Rabies virus replication in primary murine bone marrow macrophages and in human and murine macrophage-like cell lines: implications for viral persistence. *Journal of Virology* 69, 764–772.

Reagan, K.J. and Wunner, W.H. (1985) Rabies virus interaction with various cell-lines is independent of the acetylcholine-receptor – brief report. *Archieves of Virology* 84, 277–282.

Ritossa, P. and D'Agostino, S. (1967) Pathogenesis of human rabies. *Giornale di malattie infettive e parassitarie* 19, 710–732.

Ross, E. and Armentrout, S.A. (1962) Myocarditis associated with rabies – report of a case. *New England Journal of Medicine* 266, 1087–1089.

Serfaty-Lacrosniere, C., Civeira, F., Lanzberg, A., Isaia, P., Berg, J., Janus, E.D., Smith, M.P. Jr, Pritchard, P.H., Frohlich, J., Lees, R.S., Barnard, G.F., Ordovas, J.M. and Schaefer, E.J. (1994) Homozygous Tangier disease and cardiovascular disease. *Atherosclerosis* 107, 85–98.

Smith, J.S., Fishbein, D.B., Rupprecht, C.E. and Clark, K. (1991) Unexplained rabies in three immigrants in the United States. A virologic investigation. *New England Journal of Medicine* 324, 205–211.

Suja, M.S., Mahadevan, A., Madhusudana, S.N. and Shankar, S.K. (2011) Role of apoptosis in rabies viral encephalitis: a comparative study in mice, canine, and human brain with a review of literature. *Pathology Research International* 2011, 374286.

Superti, F., Seganti, L., Tsiang, H. and Orsi, N. (1984) Role of phospholipids in rhabdovirus attachment to CER cells. *Archives of Virology* 81, 321–328.

Sureau, P. (1986) Rapid technics for the laboratory diagnosis of rabies. *Archives de l'Institut Pasteur de Tunis* 63, 183–197.

Takayama, N. (2005) Clinical feature of human rabies. *Nihon rinsho. Japanese Journal of Clinical Medicine* 63, 2175–2179.

Tarr, P. and Frei, R. (2010) Molecular diagnosis of infectious diseases – a primer for general internists. *Therapeutische Umschau. Revue thérapeutique* 67, 381–386.

Thoulouze, M.I., Lafage, M., Schachner, M., Hartmann, U., Cremer, H. and Lafon, M. (1998) The neural cell adhesion molecule is a receptor for rabies virus. *Journal of Virology* 72, 7181–7190.

Toriniwa, H. and Komiya, T. (2006) Rapid detection and quantification of Japanese encephalitis virus by real-time reverse transcription loop-mediated isothermal amplification. *Microbiology and Immunology* 50, 379–387.

Tsiang, H. (1979) Evidence for an intraaxonal transport of fixed and street rabies virus. *Journal of Neuropathology & Experimental Neurology* 38, 286–299.

Tsiang, H., de la Porte, S., Ambroise, D.J., Derer, M. and Koenig, J. (1986) Infection of cultured rat myotubes and neurons from the spinal cord by rabies virus. *Journal of Neuropathology & Experimental Neurology* 45, 28–42.

Tsiang, H., Lycke, E., Ceccaldi, P.E., Ermine, A. and Hirardot, X. (1989) The anterograde transport of rabies virus in rat sensory dorsal-root ganglia neurons. *Journal of General Virology* 70, 2075–2085.

Tsiang, H., Ceccaldi, P.E. and Lycke, E. (1991) Rabies virus infection and transport in human sensory dorsal root ganglia neurons. *Journal of General Virology* 72, 1191–1194.

Tuffereau, C., Bénéjean, J., Blondel, D., Kieffer, B. and Flamand, A. (1998) Low-affinity nerve-growth factor receptor (P75NTR) can serve as a receptor for rabies virus. *EMBO Journal* 17, 7250–7259.

Tuffereau, C., Schmidt, K., Langevin, C., Lafay, F., Dechant, G. and Koltzenburg, M. (2007) The rabies virus glycoprotein receptor p75NTR is not essential for rabies virus infection. *Journal of Virology* 81, 13,622–13,630.

Ugolini, G. (1995) Specificity of rabies virus as a transneuronal tracer of motor networks: transfer from hypoglossal motoneurons to connected second-order and higher order central nervous system cell groups. *Journal of Comparative Neurology* 356, 457–480.

Ugolini, G. (2008) Use of rabies virus as a transneuronal tracer of neuronal connections: implications for the understanding of rabies pathogenesis. *Developmental Biology (Basel)* 131, 493–506.

Wacharapluesadee, S. and Hemachudha, T. (2001) Nucleic-acid sequence based amplification in the rapid diagnosis of rabies. *The Lancet* 358, 892–893.

Wakeley, P.R., Johnson, N., McElhinney, L.M., Marston, D., Sawyer, J. and Fooks, A.R. (2005) Development of a real-time, TaqMan reverse transcription-PCR assay for detection and differentiation of lyssavirus genotypes 1, 5, and 6. *Journal of Clinical Microbiology* 43, 2786–2792.

Wang, D., Coscoy, L., Zylberberg, M., Avila, P.C., Boushey, H.A., Ganem, D. and DeRisi, J.L. (2002) Microarray-based detection and genotyping of viral pathogens. *Proceedings of the National Academy of Sciences USA* 99, 15,687–15,692.

Webster, W.A., Casey, G.A. and Charlton, K.M. (1976) The mouse inoculation test in rabies diagnosis: early diagnosis in mice during the incubation period. *Canadian Journal of Comparative Medicine* 40, 322–325.

Whorton, D. (1993) What is your recommendation concerning the vaccination of an employee who will be traveling for 3 months in a country that is considered a rabies-endemic area. *Journal of Occupational and Environmental Medicine* 35, 884–885.

Wickersham, I.R., Finke, S., Conzelmann, K.K. and Callaway, E.M. (2007a) Retrograde neuronal tracing with a deletion-mutant rabies virus. *Nature Methods* 4, 47–49.

Wickersham, I.R., Lyon, D.C., Barnard, R.J., Mori, T., Finke, S., Conzelmann, K.K., Young, J.A. and Callaway, E.M. (2007b) Monosynaptic restriction of transsynaptic tracing from single, genetically targeted neurons. *Neuron* 53, 639–647.

Wilde, H. and Lumlertdacha, B. (2011) Rabies research in resource-poor countries. *Advances in Virus Research: Research Advances in Rabies* 79, 449.

Yousaf, M.Z., Qasim, M., Zia, S., Khan, Mu., Ashfaq, U.A. and Khan, S. (2012) Rabies molecular virology, diagnosis, prevention and treatment. *Virology Journal* 9, 50.

Zaytseva, N.V., Montagna, R.A., Lee, E.M. and Baeumner, A.J. (2004) Multi-analyte single-membrane biosensor for the serotype-specific detection of Dengue virus. *Analytical and Bioanalytical Chemistry* 380, 46–53.

14 Ebolavirus

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14.1 Introduction

14.1.1 Summary

The ebolavirus is a zoonotic *Filoviridae* and causative agent of severe Ebola haemorrhagic fever (EHF) in primates, respiratory syndromes in swine, subclinical persistent infections in bats and asymptomatic diseases in canines. The high mortality rate in humans and non-human primates (NHPs) observed in periodic outbreaks in sub-Saharan Africa indicates that ebolaviruses remain one of the deadliest zoonotic pathogens. In primates, the clinical aspect of the disease is nearly indistinguishable from marburgvirus infection, another member of the *Filoviridae*. In addition to poaching, ebolaviruses contribute to the decimation of the gorilla and chimpanzee populations in sub-Saharan Africa. The ebolavirus is an agricultural concern in Southeast Asia, primarily in swine. Humans are infected with ebolavirus by close contact with blood, secretions, aerosols, organs or other bodily fluids from infected animals. In Africa, human infections have been documented through the handling of infected chimpanzees, gorillas, fruit bats, monkeys, forest antelope (duiker) and porcupines found dead or ill in rainforests and the co-habitation with bats in enclosed areas, such as caves and infested factories. Once introduced into the human population, the virus spreads via human-to-human transmission primarily through contact with bodily fluids. The manifestations of this disease are similar in humans and NHPs, and consist of acute viral haemorrhagic disease symptoms, including high levels of viraemia, widespread tissue destruction, severe coagulation abnormalities, and significant morbidity and mortality (ranging from 23% to 100%). Accumulating evidence has revealed a role for fruit bats of the *Pteropodidae* family as the natural hosts of ebolaviruses. However, the characterization of the enzootic cycle of ebolaviruses remains an area of active research. Filoviruses are highly infectious and contagious, and appropriate precautions must be taken to protect personnel when collecting, transporting or handling samples. The World Health Organization classifies the ebolavirus as a Risk Group 4 Pathogen, requiring biosafety level 4 (BSL-4)-equivalent containment. Currently, there are no vaccines or therapeutics licensed for the prevention or treatment of this disease in humans or animals.

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14.1.2 History

The family *Filoviridae* consists of enveloped, non-segmented, negative-strand RNA viruses (Kuhn *et al.*, 2010) that form part of a larger order *Mononegavirales*, which includes other virus families with similar genomic characteristics and organization (Feldmann *et al.*, 2005). The *Filoviridae* contains three genera (Kuhn *et al.*, 2012): *Ebolavirus* and *Marburgvirus*, which cause zoonotic diseases, and the recently proposed genus *Cuevavirus*, which infects bats. The genus *Ebolavirus* consists of five species (each with a single virus): Zaire ebolavirus (EBOV), Sudan ebolavirus (SUDV), Reston ebolavirus (RESTV), Taï Forest ebolavirus (TAFV) and Bundibugyo ebolavirus (BDBV). Within each species, one or more ebolavirus isolates correspond to specific outbreaks. These viruses, except for RESTV, cause acute and highly lethal EHF in humans. In contrast, RESTV causes subclinical or asymptomatic infections in humans, but induces severe EHF in macaques (Kurosaki and Yasuda, 2011). Ebolavirus outbreaks in humans occur in central and western African rain forests (Kuhn, 2008), and disease outbreaks in Asia have been associated with RESTV infection in macaques, pigs and bats. In the USA and Italy, RESTV outbreaks in macaques have been linked to animals imported from the Philippines.

Ebolaviruses were first recognized in two 1976 human outbreaks in the Democratic Republic of the Congo (DRC; formerly known as Zaire), resulting from EBOV infections (WHO, 1978b) and in Sudan, resulting from infections with SUDV (WHO, 1978a) (Table 14.1). Multiple outbreaks were observed from 1977 to 1979 in sub-Saharan Africa, with case fatality rates ranging from 53% to 88%. There were no known major outbreaks for 15 years. However, disease outbreaks in humans associated with ebolavirus activity in animals have occurred more frequently in the last 20 years (Table 14.1), including the recent 2012 outbreaks in the DRC and Uganda. EBOV and SUDV infections are the most common causes of disease outbreaks. In 1994, TAFV emerged, causing a single non-fatal human case contracted during an autopsy of a wild chimpanzee in the Taï Forest, Côte d'Ivoire (Le Guenno *et al.*, 1995). In 2007, BDBV emerged in the Bundibugyo district of Western Uganda, causing an outbreak that included 149 suspected human cases with 37 deaths (25% case fatality rate) (Towner *et al.*, 2008). BDBV is the most likely the source of a current ongoing outbreak in Orientale Province, DRC, resulting in multiple human deaths. RESTV was first isolated in 1989 at an animal quarantine facility in Reston, Virginia, USA from dead and dying cynomolgus macaques (*Macaca fascicularis*) (Jahrling *et al.*, 1990) imported from the Philippines (Hayes *et al.*, 1992). Additional RESTV epizootics in cynomolgus macaques in 1989, 1990, 1992 and 1996 were also attributed to NHPs imported from the Philippines (Morikawa *et al.*, 2007). To prevent the re-occurrence of RESTV outbreaks from imported monkeys, the Foreign Quarantine Regulations for the importation of NHPs (42CFR-Part71.53) was established in 1990, which requires a mandatory 31-day quarantine and testing period for imported African green, cynomolgus and rhesus macaques. In 2008, RESTV was detected in domestic swine from the Philippines co-infected with porcine reproductive and respiratory syndrome virus (PRRSV), an arterivirus (Barrette *et al.*, 2009). Evidence was originally based on PCR results but was later confirmed by virus isolation (Miranda and Miranda, 2011). Anti-RESTV antibodies were detected in Philippine pig farm and slaughterhouse workers, suggesting pig-to-human transmission without evidence of disease (WHO, 2009).

14.2 Ebolavirus Biology

14.2.1 Structure, genome organization and viral proteins

Ebolavirus particles are filaments that assume various forms, including shepherd's

Table 14.1. Ebolavirus outbreaks[a].

Year	Country	Ebolavirus species	Human cases	Human deaths	Human case fatality
2012 (Dec–)[b]	Uganda	Unconfirmed	7[c]	4[c]	57%[c]
2012 (Nov–)[b]	Democratic Republic of Congo	BDBV	77[c]	36[c]	47%[c]
2012 (Jul–Oct)	Uganda	Unconfirmed	24	17	71%
2011	Uganda	SUDV	1	1	100%
2008	Philippines	RESTV	6[d]	0	0
2008	Democratic Republic of Congo	EBOV	32	14	44%
2007	Uganda	BDBV	149	37	25%
2007	Democratic Republic of Congo	EBOV	264	187	71%
2005	Congo	EBOV	12	10	83%
2004	Sudan	SUDV	17	7	41%
2003 (Nov–Dec)	Congo	EBOV	35	29	83%
2003 (Jan–Apr)	Congo	EBOV	143	128	90%
2001–2002	Congo	EBOV	59	44	75%
2001–2002	Gabon	EBOV	65	53	82%
2000	Uganda	SUDV	425	224	53%
1996	South Africa (ex-Gabon)	EBOV	1	1	100%
1996 (Jul–Dec)	Gabon	EBOV	60	45	75%
1996 (Jan–Apr)	Gabon	EBOV	31	21	68%
1996	US	RESTV	0	0	0
1996	Philippines	RESTV	0	0	0
1995	Democratic Republic of Congo	EBOV	315	254	81%
1994	Cote d'Ivoire	TAFV	1	0	0%
1994	Gabon	EBOV	52	31	60%
1992	Italy	RESTV	0	0	0
1990	US	RESTV	4[§]	0	0
1989–1990	Philippines	RESTV	3[§]	0	0
1989	US	RESTV	0	0	0
1979	Sudan	SUDV	34	22	65%
1977	Democratic Republic of Congo	EBOV	1	1	100%
1976	Sudan	SUDV	284	151	53%
1976	Democratic Republic of Congo	EBOV	318	280	88%

[a] Modified from CDC as of 31 July 2012; [b] estimated from ongoing outbreaks as of January 2013; [c] partial counts from ongoing outbreaks; [d] asymptomatic.

crook, 'U', '6', coiled, toroid or branched shapes (Fig. 14.1), which are generally 80 nm in width and vary widely in length (974–1086 nm). The viral genome consists of a single-stranded, linear, 19 kb, non-segmented, negative-sense RNA. A reverse genetics system that allows the rescue of infectious virus from eukaryotic cells requires the transfection of multiple plasmids encoding genomic RNA and messages of viral proteins (Muhlberger *et al.*, 1999; Volchkov *et al.*, 2001).

Ebolavirus contains seven structural proteins that form the ribonucleoprotein (RNP) complex or are associated with the lipid envelope of the virion (Richardson *et al.*, 2010; Bradfute *et al.*, 2011) (Fig. 14.1). The RNA is encased in a RNP complex consisting of the nucleoprotein (NP),

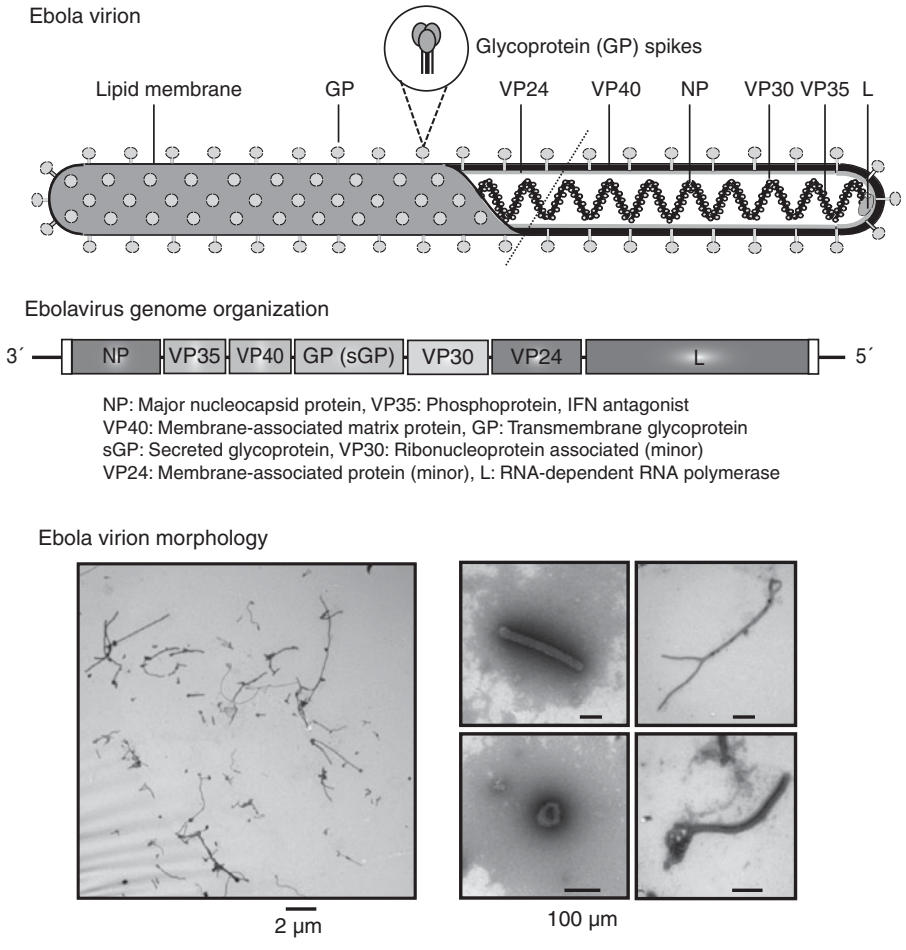

Fig. 14.1. Structure, genome organization and morphology of Ebolavirus. (Electron micrographs were kindly provided by Dr Anna Honko and Chris Reed.)

viral proteins VP35 and VP30 and the RNA-dependent RNA polymerase or L protein (Regnery *et al.*, 1980; Sanchez *et al.*, 2007), all of which are required for viral transcription and replication (Mühlberger *et al.*, 1998, 1999; Mühlberger, 2007). The NP protein is the major phosphoprotein surrounding the viral RNA and forming the nucleocapsid. VP35 serves as a bridge that connects NP and L and acts as a polymerase co-factor (Becker *et al.*, 1998; Muhlberger *et al.*, 1999). VP30 functions as an activator and regulator of ebolavirus transcription and is involved in nucleocapsid assembly (Mühlberger, 2007).

The structural envelope-associated proteins include VP24, VP40 and the viral glycoprotein (GP). VP40, the most abundant protein in the virion, is a matrix protein that plays a role in viral assembly and budding (Feldmann *et al.*, 1992; Sanchez *et al.*, 1993; Bavari *et al.*, 2002). VP24 is a minor matrix protein that plays an unclear role in assembly and budding (Han *et al.*, 2003; Bamberg *et al.*, 2005). GP is a structural protein that forms homotrimeric spikes that are inserted into the lipid envelope and that mediate the binding of the virus to cellular receptors and fusion of viral and cellular membranes. Subsequent to cell entry, the viral genome is released into the cytoplasm, followed by

the disassembly of the RNP complex and the initiation of viral replication. Transcriptional editing of the GP gene from ebolavirus and cuevavirus, but not marburgvirus, results in the expression of three partially overlapping proteins that share the first N-terminal 295 amino acids: sGP/D-peptide, GP and ssGP (Mehedi *et al.*, 2011 and references therein). The primary product of the unedited ebolavirus GP gene is sGP/D-peptide, a protein of 324 amino acids abundantly produced during infection (Volchkov *et al.*, 1995; Sanchez *et al.*, 1996). The sGP/D-peptide accounts for approximately 70% of the total expression of the GP gene (Mehedi *et al.*, 2011), lacks a transmembrane anchor, forms disulfide-linked homodimers (Feldmann and Kiley, 1999), and is cleaved by furin into mature sGP and the D-peptide. Transcriptional editing of the GP gene at a series of seven uridine residues results in the addition of two adenosine residues (+2 shift), which reduce the open reading frame (ORF) to a soluble protein of 298 amino acids, termed the ssGP glycoprotein, and accounts for approximately 2–6% of the total GP content (Mehedi *et al.*, 2011). The GP membrane-bound protein is expressed upon transcriptional editing of the GP gene at the same series of seven uridine residues, resulting in the addition of only one adenosine residue. This transcriptional editing causes a +1 shift that extends the ORF to transcribe a GP protein of 676 amino acid residues. GP is a class I integral membrane glycoprotein that undergoes complex post-translational processing involving furin cleavage and disulfide-bond formation between the N-terminus and the membrane proximal portion of the GP. The mature transmembrane GP present on the viral envelope and membrane of infected cells is a homotrimer formed by two subunits: GP1 and GP2. The membrane-anchored GP2 is covalently linked via disulfide linkage to the N-terminus of GP1, which contains a highly O-glycosylated mucin-like domain (Volchkov *et al.*, 1998; Jeffers *et al.*, 2002). A significant amount of GP1 is shed from the cells after release from the GP2 subunit. The GP protein is required for cell entry, but the function of the other products of the GP gene is unclear; however, these proteins might facilitate the evasion of host immune responses.

14.2.2 Cell entry and replication

Numerous host proteins have been identified as candidate Filovirus cell-surface receptors, such as transferrin, DC-SIGN and HAVCR1 (Alvarez *et al.*, 2002; Richardson *et al.*, 2010; Kondratowicz *et al.*, 2011), and the NPC1 protein has recently been described as a significant intracellular host entry factor (review in White and Schornberg, 2012). Filoviruses use ubiquitous cellular receptors that mediate the infection of a wide variety of cell types in the infected host. After entry, Filoviruses replicate their genomes and translate viral proteins in the cytoplasm. At least two viral proteins, VP24 and VP35, have been shown to block host interferon (IFN) responses (Basler and Amarasinghe, 2009) and might significantly influence pathogenesis.

14.3 Pathogenesis

14.3.1 Virus transmission

The first identified ebolavirus outbreak occurred in Sudanese workers from a single cotton factory in 1976. Thereafter, most human ebolavirus outbreaks have been associated with infected NHPs or bats in cotton factories, caves and mines (review in Warfield *et al.*, 2009). Bats are currently considered to be the natural hosts for all Filoviruses. Although anti-ebolavirus antibodies have been detected in bats during field surveillance studies, thus far, ebolaviruses have not been isolated from infected bats in nature. Infected bats could spread the virus directly to NHPs and humans via aerosols and excretions. In primates, ebolavirus infection results in severe haemorrhagic fever with high levels of morbidity and mortality. Animal mortality preceded human ebolavirus outbreaks in Gabon and the Republic of Congo, which

have been linked to contact with dead monkeys, gorillas (*Gorilla gorilla*), chimpanzees (*Pan troglodytes*) and duikers (*Cephalophus* spp.) (Leroy *et al.*, 2004). Close contact with infected individuals is responsible for outbreaks in the human population and perhaps NHP colonies. In the Philippines, the natural infection of pigs with RESTV was associated with the seroconversion of animal caretakers (Barrette *et al.*, 2009), suggesting that pigs could also be naturally infected with ebolavirus strains that are pathogenic to humans. This hypothesis was recently confirmed in pigs experimentally infected with EBOV, which developed severe respiratory syndromes and transmitted the disease to cohabiting pigs (Kobinger *et al.*, 2011) and NHPs (Weingartl *et al.*, 2012).

The ecology of ebolavirus is poorly understood, and it is possible that other animals are also involved in the transmission of this virus. For instance, dogs that eat infected carcasses and come into contact with humans develop asymptomatic infection and seroconvert. However, it is unknown whether dogs can directly transmit ebolavirus to humans (Allela *et al.*, 2005).

14.3.2 Clinical outcomes

The natural infection of primates, swine, canines and bats with ebolavirus results in different outcomes, ranging from high morbidity and mortality to asymptomatic infections. The experimental infection of guinea pigs (Ryabchikova *et al.*, 1996), goats (Dedkova *et al.*, 1994), horses (Krasnianskii *et al.*, 1994) and laboratory animals with wild-type ebolavirus induces mild symptoms or asymptomatic infection, but infections of these animals have not been detected in nature. The passage of ebolavirus in mice and guinea pigs resulted in the selection of lethal variants that recapitulate some aspects of the ebolavirus infection in primates. These mouse and guinea pig challenge models of ebolavirus infection have been extensively characterized and reviewed (Sanchez *et al.*, 2007) and will not be the focus of this chapter.

In primates, ebolavirus infection primarily results in symptomatic cases, but serological surveys indicate that asymptomatic cases rarely occur. Humans and NHPs develop similar clinical manifestations of EHF. Ebolavirus infection has been extensively studied in NHPs, which are used as a model for the development of vaccines and therapeutics. The incubation period in primates can vary but is generally 3–9 days (range 3–21 days). There is a relatively low concentration of the virus in blood and tissues during the early phase of the incubation period. Clinical signs of the disease are observed when the virus spreads to multiple tissues and titres in blood significantly increase, resulting in high rates of transmission. The disease presents abruptly with non-specific flu-like symptoms, such as fever (as high as 39°C), myalgia and malaise, including chills, headache and sore throat (Egbring *et al.*, 1971; Martini, 1971; Gear *et al.*, 1975; Gear, 1989; Borio *et al.*, 2002). Vomiting, abdominal pain, watery diarrhoea, anorexia, dyspnoea and dysphagia are often reported in humans and have been observed in macaques. A high fever usually persists during the illness, followed by progressive and rapid debilitation to more severe symptoms associated with EHF. Bleeding and coagulation abnormalities, including gastrointestinal bleeding, rash and a range of haematological irregularities, such as lymphopenia and neutrophilia, are observed in severe cases. Cytokines are released during infection, which exaggerate inflammatory responses and cause some of the symptoms associated with the disease. As the disease progresses, the gastrointestinal tract, respiratory tract, and vascular and neurologic systems are affected. The virus eventually infects microvascular endothelial cells and compromises vascular integrity. A maculopapular rash might be visible on the axilla, groin, forehead and trunk (chest, back, stomach). Damage to the liver combined with massive viraemia leads to disseminated intravascular coagulopathy. The involvement of the central nervous system results in confusion, irritability and aggression. In more severe and fatal cases, symptoms increase and include diffuse bleeding

from multiple organs and venipuncture sites, hypotensive shock, sustained high fever, the development of prostration, tachypnoea, anuria and jaundice potentially from liver destruction. Blood chemistry reflects elevated levels of transaminase, amylase, creatinine and blood urea nitrogen, indicating kidney damage. Death occurs during the second week, with a median interval of 8 days (range 2–16 days), from multiple organ failure. The fatality rate ranges from 20% to 90%, but the availability of adequate supportive health care can improve survival. By the end of the first week, symptomatic patients who will survive the infection begin to improve, and signs of coagulopathy generally are limited to conjunctival haemorrhages, easy bruising and bleeding from venipuncture sites; viraemia also begins to diminish (Mahanty and Bray, 2004). Recovery from the disease is prolonged, and secondary infections have been observed in animal models of the disease, likely reflecting impaired immune responses after virus infection.

Ebolavirus-infected bats have not been isolated yet in nature, but there is significant evidence indicating that some wild bats have anti-ebolavirus antibodies. In experimentally infected bats, ebolavirus replicates in lung endothelial cells, and the animals develop viraemia with limited signs of disease, shed the virus in their faeces and survive the infection (Swanepoel *et al.*, 1996; Leroy *et al.*, 2004).

Although pigs co-infected with circovirus and RESTV develop severe respiratory syndrome, pigs experimentally infected with RESTV only develop subclinical disease, suggesting that the respiratory syndrome was enhanced or caused by the circovirus co-infection (Marsh *et al.*, 2011). However, pigs experimentally infected with EBOV through mucosal exposure develop a severe respiratory syndrome with lung pathology, producing high titres of virus in the respiratory tract and shedding virus from the oronasal mucosa (Kobinger *et al.*, 2011).

In summary, natural ebolavirus infections result in different diseases, depending on the host and virus strain, ranging from unapparent disease in bats and dogs, respiratory or subclinical disease in pigs, to EHF in humans and NHPs with high levels of morbidity and mortality.

14.3.3 Histopathology

In primates, ebolavirus replicates in multiple tissues, resulting in the pathogenic process that leads to EHF (review in Warfield *et al.*, 2009). EBOV replicates in macrophages and dendritic cells, which exhibit impaired function early during infection. The virus infection spreads throughout the body, infecting key organs, including the liver, kidneys, spleen and lungs. Lymphocytes are not infected, but NK and T-cells undergo bystander apoptosis, the proportion of neutrophils is increased and circulating B cells remain constant. Late in infection, thrombocytopenia and the infection of endothelial cells results in leakage from the blood compartment. Necropsy and histological analyses reveal minor lesions during the first 48 hours of infection, with increased pathology in the lymph nodes, tonsils, intestine, liver (Fig. 14.2A,B,C), kidneys, spleen and lungs, and petechiae (Geisbert *et al.*, 2003a).

Pigs experimentally infected with RESTV develop a subclinical respiratory infection without gross pneumonic lesions, the virus replicates in the lungs, lymphoid tissues and nasopharynx, and there is occasional faecal shedding (Marsh *et al.*, 2011). However, experimental infection of pigs with EBOV induced severe respiratory disease with histological pathogenic changes in the lungs and associated lymph nodes; airway inflammation; significant viral replication in macrophages, pneumocytes and lung endothelial cells; moderate virus replication in the bladder, heart, tonsils, muscle, liver and gut; and a lack of coagulation abnormalities (Kobinger *et al.*, 2011).

Fruit bats experimentally inoculated with EBOV developed asymptomatic infection (Swanepoel *et al.*, 1996), and the virus was detected in blood, viscera and faeces. The histopathological analysis of lung tissue from one insectivorous bat stained with anti-ebolavirus antibodies revealed the

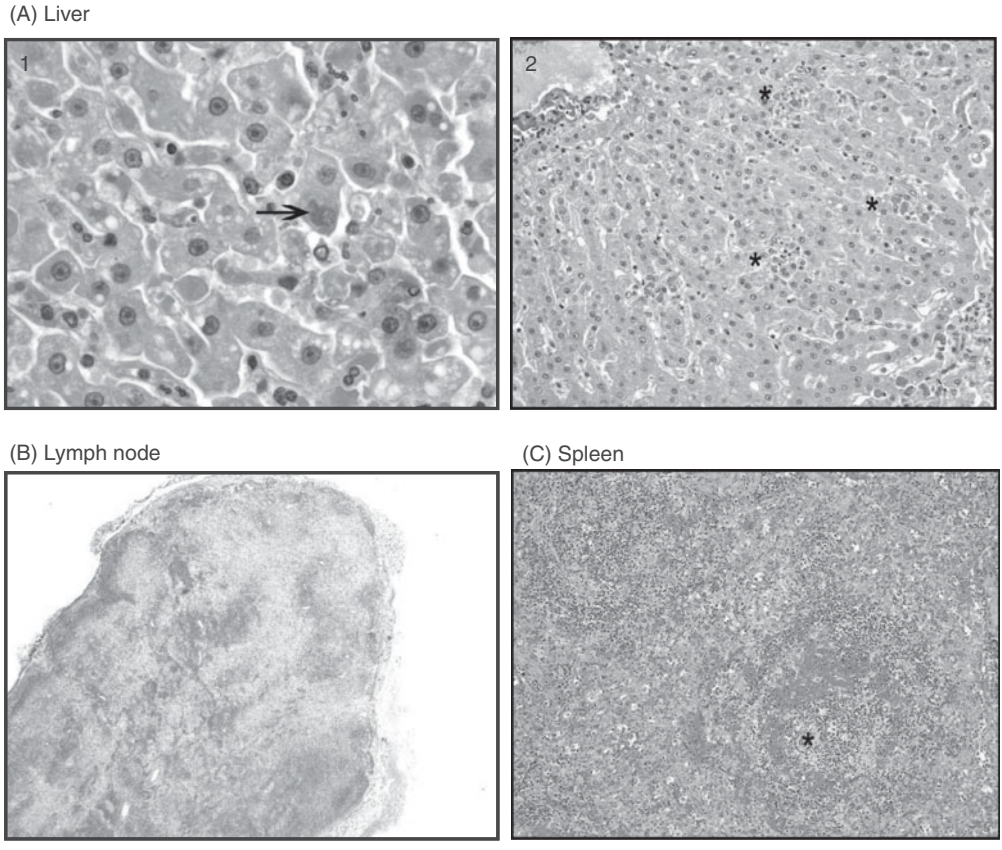

Fig. 14.2. Profound lymphoid destruction caused by infection. (A) Diffuse marked infection of the liver. Liver 1: Filoviral intracytoplasmic inclusion bodies in numerous hepatocytes (arrow). Liver 2: Random multifocal degeneration and necrosis of hepatocytes (asterisks) with disruption of normal hepatic cord architecture. (B) TB LN: marked destruction of lymph node, diffuse apoptosis and loss lymphocytes. Accumulation of fibrin and multifocal haemorrhage. (C) Spleen: diffuse loss of white pulp. Marked apoptosis of lymphocytes within germinal centres (asterisk) and loss of lymphocytes and haemorrhage within the surrounding mantle layer. Marked fibrin accumulation in both the red and white pulp. (Histology kindly provided by LTC Nancy A. Twenhafel, USAMRIID Pathology Division.)

presence of ebolavirus antigens in endothelial cells (Swanepoel *et al.*, 1996), further suggesting that insectivorous bats could also be a virus reservoir.

Little is known about the histopathology in other naturally infected animal species.

14.3.4 Immune responses

In fatal infections of primates, ebolavirus rapidly cripples the immune system by targeting cells of the innate immune system, blocking the activation of cellular and humoral immunity (review in Mohamadzadeh *et al.*, 2007). Dendritic cells (DCs) are often the first cells to become infected (Geisbert *et al.*, 2003a; Mohamadzadeh *et al.*, 2007), which fail to mature and are incapable of producing the appropriate cytokines for T-cell activation and signaling. Ebolavirus VP24 and VP35 proteins block IFN production in infected cells, including plasmacytoid DCs, which are required for the efficient activation of natural killer (NK) cells, invariant NK T-cells (iNKT cells) and

T-cells. Although ebolavirus does not infect T-cells, macrophages infected with the virus secrete inappropriate cytokines, resulting in the anergy and apoptosis of T-cells, thereby preventing the induction of the acquired immune response. Circulating, infected monocytes express large amounts of tissue factor, which initiates disseminated intravascular coagulation and tissue damage. This damage continues as the viral load increases during the course of the disease, and the primate is soon overwhelmed, can no longer contain the infection and dies from EHF within a few days. In non-fatal cases with different degrees of morbidity, the host's immune system is capable of mounting innate and adaptive immune responses, which result in virus-specific antibody production and cellular immune responses that control and clear virus infection.

The subclinical disease in pigs experimentally infected with RESTV via the oronasal route is characterized by viral replication in the lungs and lymphoid tissues, virus shedding from the nasopharynx, seroconversion at 8–12 days post-infection and clearance of the virus (Marsh *et al.*, 2011). The infiltration of alveolar macrophages in the lung and rapid seroconversion suggest non-compromised innate and adaptive immune responses in RESTV-infected pigs. A more severe respiratory syndrome develops in EBOV-infected pigs with significant lung lesions. The down-regulation of IFN-α, increased inflammatory cytokines and delayed seroconversion by day 21 post-infection (Kobinger *et al.*, 2011) suggest that EBOV might also target the innate and adaptive responses in swine to a lesser extent than in primates.

Ebolavirus infection results in seroconversion in bats and dogs, but there are limited data concerning other aspects of the immune response, and there are no data available on the immune responses in other naturally infected animals.

14.4 Clinical Pathology

The first symptoms of ebolavirus infection in primates are non-specific and include fever, chills, anorexia, vomiting, cough, arthralgia and diarrhoea. Infected primates become dehydrated, apathetic and disoriented. At 5–10 days after the onset of the disease, primates develop EHF symptoms, including a maculopapular rash on the trunk and limbs, bleeding from the gastrointestinal and urogenital tracts, petechiae and haemorrhaging from mucous membranes.

The subclinical disease in pigs inoculated with RESTV via the oronasal or subcutaneous routes does not result in gross pneumonic lesions, with viral replication in the lungs, lymphoid tissues and nasopharynx. There is occasional faecal shedding, and seroconversion and clearance of the virus occurs 10–12 days post-infection (Marsh *et al.*, 2011). However, pigs experimentally infected with EBOV develop a severe respiratory syndrome without the characteristic coagulopathy observed in primates (Barrette *et al.*, 2009). EBOV-infected pigs develop macroscopic pathogenic changes in the lungs and associated lymph nodes, reduced viraemia and minimal or no replication of the virus in other organs (Kobinger *et al.*, 2011).

Bats experimentally inoculated with EBOV develop asymptomatic infection (Swanepoel *et al.*, 1996). Little is known about the disease in other naturally infected animal species.

14.5 Diagnosis of Ebolavirus Infection

The early symptoms of ebolavirus infection are common to many other diseases. Therefore, diagnosis of ebolavirus infection relies on laboratory tests, including the isolation of the virus during the acute phase of the disease, identification of viral antigens, amplification of viral nucleic acids and the detection of ebolavirus-specific immune responses during the acute, convalescent and post-convalescent phases. Sensitive and specific methods have been developed to diagnose infection using blood, oral/nasal swabs (Towner *et al.*, 2007), post-mortem tissues and excreta. Specimens should be handled with extreme care due to their possible infectious nature. Some diagnostic tests have been deployed to laboratories in the field, which are essential for the detection and management of outbreaks. Currently, there are no licensed

tests available; thus, confirmation of ebolavirus infection in shipped samples is conducted at reference laboratories under BSL-4 conditions, such as the CDC in Atlanta, USA. However, the logistics of transporting the samples and handling potentially infectious materials adds significant time to the diagnostic process. The following tests have been developed to diagnose ebolavirus infection in humans and animals.

14.5.1 Virus isolation

Virus isolation is the ebolavirus diagnostic gold standard but requires BSL-4 containment, which is not available in field laboratories, and the proper handling of samples to preserve virus infectivity. During the short acute phase of the disease, ebolavirus can be isolated from blood, serum or other clinical samples by infecting African green monkey kidney Vero E6 cells, Rhesus monkey kidney MA-104 cells, human adrenal carcinoma SW13 cells, or permissive primary cell cultures. The identification of the virus replicating in cell culture is commonly performed using immunofluorescence assays with anti-ebolavirus antibodies (Rodriguez *et al.*, 1999). Inoculation into guinea pigs or serial passages in cell culture might be required for viral isolation because field isolates do not always induce a cytopathic effect. The fluorescence focus assays (FFA) is more efficient than the direct plaque assay for the detection of ebolavirus in field samples (Ksiazek *et al.*, 1999a). Animals that succumb early in infection exhibit high viraemia levels, whereas animals that survive develop anti-ebolavirus antibodies that could prevent virus isolation. Consequently, virus isolation is only possible during the early acute phase before the onset of the antibody response.

14.5.2 Viral antigen tests

High titres of virus and antigens are present early in infection, which are used to diagnose ebolavirus infection. Humans and NHPs that succumb to EBOV infection develop low or no antibody response; therefore, antigen detection is particularly important to confirm infection in fatal cases. Capture ELISA has been developed to detect ebolavirus antigens in blood, serum and tissue suspensions under BSL-4 conditions and in gamma-irradiated samples under BSL-2 conditions. An ebolavirus antigen test was developed at the CDC (Ksiazek *et al.*, 1992) and is broadly used to analyse outbreak samples. This test is based on the capture of viral antigens on microtitre plates coated with a mixture of eight anti-ebolavirus monoclonal antibodies produced against EBOV and SUDV, detection of the ebolavirus captured antigens with hyper-immune rabbit anti-EBOV antibodies raised against purified virus, and staining with horseradish peroxidase-labelled affinity-purified goat anti-rabbit antibody and ABTS substrate. This antigen detection test has approximately 98% sensitivity and specificity compared with the virus isolation gold standard. For EBOV, the limit of detection of the antigen capture ELISA is approximately 10^4 PFU/ml, corresponding to approximately 10^7 RNA copies/ml through quantitative reverse transcriptase polymerase chain reaction (RT-PCR) (Towner *et al.*, 2004). However, there is significant variation in the particle-to-PFU ratio of other ebolavirus strains. Several antigen-capture assays based on monoclonal and polyclonal antibodies raised against ebolavirus or recombinant proteins have been developed for GP (Lucht *et al.*, 2004; Yu *et al.*, 2006; Ou *et al.*, 2011), NP (Niikura *et al.*, 2001; Ikegami *et al.*, 2003a; Goodchild *et al.*, 2011) and VP40 (Lucht *et al.*, 2003; Goodchild *et al.*, 2011). These antigen assays exhibit different degrees of sensitivity, specificity and cross-reactivity between ebolavirus species and have not been fully validated in outbreak settings.

14.5.3 Nucleic acid tests

The detection of ebolavirus genomic and messenger RNAs using RT-PCR assays is commonly used to diagnose infection and further characterize the species and strain

of virus. PCR assays are highly sensitive and can detect ebolavirus 24–48 hours before antigen assays (Towner *et al.*, 2004) and viral RNA several weeks after the acute disease. Highly sensitive and specific RT-PCR assays have been developed to diagnose ebolavirus infection. However, these assays have drawbacks that are inherent to PCR technology, primarily reflecting the variability of the ebolavirus RNA genome (32–45% nucleotide differences; Towner *et al.*, 2008), which result in false negatives due to primer mismatches, and contaminations, which result in false positives. Therefore, the confirmation of the RT-PCR diagnostic results requires additional assays, such as antigen detection, virus isolation and seroconversion.

Nucleic acids for ebolavirus diagnostics are extracted from blood, other body fluids, mucosal swabs and tissues with chaotropic agents, such as guanidinium thiocyanate, phenol-chloroform and detergents, in sequential steps or in one mixture (Chomczynski and Sacchi, 1987) using, for example, TRIzol (Invitrogen) or Tripure (Roche) reagents. This treatment inactivates the virus and facilitates field diagnosis under BSL-2 conditions. Primers in conserved regions of the L and GP genes (Table 14.2) have been used to detect almost all species of ebolavirus (Sanchez *et al.*, 1999) in several outbreaks (Sanchez *et al.*, 1999; Leroy *et al.*, 2000; Drosten *et al.*, 2002). However, the sensitivity of the RT-PCR assays was low, and these primer sets did not detect the first outbreak of BDBV, the new species that emerged in 2007 (Towner *et al.*, 2008). The redesign of primer sets targeting the NP region can now detect all known species of ebolavirus (Ogawa *et al.*, 2011). Nested PCR assays with 10-fold higher sensitivity and sensitive species-specific quantitative-RT-PCR (Q-RT-PCR) assays have been developed using one-step RT-PCR kits, which simplify the detection and titration of ebolavirus nucleic acids (Towner *et al.*, 2004, 2007; Panning *et al.*, 2007; Stephens *et al.*, 2010; Trombley *et al.*, 2010).

The apparent discrepancy between the sensitivity of the PCR assays is likely due to the particle-to-PFU ratio that varies between species of ebolavirus. For EBOV, the assessment of the particle-to-PFU ratio revealed that one PFU corresponds to 1000–10,000 genomes detected through Q-RT-PCR (Towner *et al.*, 2004). However, for TAFV and RESTV, the particle-to-PFU ratios were approximately 600:1 and 35:1, respectively (Trombley *et al.*, 2010).

In addition to RT-PCR, nucleic acid deep sequencing technology has been used to diagnose ebolavirus infection in cases where specific PCR amplicons were not detected in the suspected samples (Towner *et al.*, 2008) or great sequence diversity was observed in PCR amplicons (Negredo *et al.*, 2011). Deep sequencing technology is advancing rapidly, and costs are decreasing significantly; thus, it is tantalizing to speculate that this technique could be used routinely to diagnose viral infections in the future.

14.5.4 Immunohistochemistry, *in situ* hybridization and histopathological examination

Biopsies and necropsies from euthanized animals or carcasses found in the field have been used to diagnose ebolavirus infection (Zaki *et al.*, 1999; Geisbert *et al.*, 2003c; Larsen *et al.*, 2007 and references therein). These assays facilitate the detection of viral antigen within formalin-fixed tissues, which inactivates ebolavirus and allows the handling of the samples under BSL-2 conditions. The diagnosis based on histology is laborious and requires specialized labs but provides confirmatory value and has been used to verify infections in a wide variety of animals (Swanepoel *et al.*, 1996; Rouquet *et al.*, 2005; Barrette *et al.*, 2009; Kobinger *et al.*, 2011 and references therein).

14.5.5 Immunoelectron microscopy

The detection of ebolavirus particles through negative staining and immunoelectron microscopy (EM) in tissues, fluids and infected

Table 14.2. Examples of RT-PCR assays to diagnose ebolavirus infection.

Technique	Specificity	Gene target	Amplicon Size (bp)	Primers 5′ to 3′ [name, (polarity), sequence]	Sensitivity RNA copies or (PFU)/ reaction	Source
Single-round RT-PCR	EBOV, RESTV, SUDV, TAFV, MARV	L	419	**FILO-A** (+) ATCGGAATTTTTCTTTCTCATT **FILO-B** (–) ATGTGGTGGGTTATAATAATCACTGACATG	100[a]	Sanchez *et al.* (1999)
	EBOV, RESTV, SUDV, TAFV	GP	580	**EBO-BP1** (+) AATGGGCTGAAAATTGCTACAATC **EBO-GP2** (–) TTTTTTTAGTTTCCCAGAAGGCCCACT	<100[a]	Sanchez *et al.* (1999)
	BDBV, EBOV, RESTV, SUDV, TAFV	NP	594	**FiloNP-Fe** (+) TGGCAATCAGTDGGACACATGATGGT **FiloNP-Re** (–) GAAGCTGATTTCRTTCTTYTTCTGATGGAA	(0.001)	Ogawa *et al.* (2011)
	RESTV	NP	337	**RES-NP1** (+) GTATTTGGAAGGTCATGGATTC **RES-NP2** (–) CAAGAAATTAGTCCTCATCAATC	>100[a]	Sanchez *et al.* (1999)
	EBOV	NP	268	**ZAI-NP1** (+) GGACCGCCAAGGTAAAAAATGA **ZAI-NP2** (–) GCATATTGTTGGAGTTGCTTCTCAGC	>100[a]	Feldmann *et al.* (1992) Sanchez *et al.* (1999)
Nested RT-PCR	SUDV, EBOV	NP	First round 150 Second round 130	**SudZaiNP1** (+) GAGACAACGGAAGCTAATGC **SudZaiNP1** (–) AACGGAAGATCACCATCATG **SudZaiNP2** (+) GGTCAGTTTCTATCCTTTGC **SudZaiNP2** (–) CATGTGTCCAACTGATTGCC	10	Towner *et al.* (2004)
One-step Q-RT-PCR	BDBV	NP	74	**EboU965** (+) GAGAAAAGGCCTGTCTGGAGAA **EboU1039** (–) TCGGGTATTGAATCAGACCTTGTT **Probe** FAM-TTCAACGACAAATCCAAGTGCACGCA-BHQ1	Sensitive but undetermined	Towner *et al.* (2008)
	TAFV	NP	74	**F2016** (+) ATGGAAACCAAGGCGAAACTG **R2089** (–) TACTTGTGGCATTGGCTTGTCT **p2045S** 6FAM-CGGGTAGCCCCCAAC-MGBNFQ	Sensitive but undetermined	Trombley *et al.* (2010)
	RESTV	GP	64	**F1123** (+) CCCATCTCCGCCCACAA **R1186** (–) GAGTGGAATCCTCTGAAACCAATT **p1143S** 6FAM-CGCAGGCGAAGAC-MGBNFQ	586 (1.0)	Trombley *et al.* (2010)
	SUDV	GP	79	**F564** (+) TGTACACAAAGTCTCAGGAACTGG **R642** (–) GTCATACAGGAAGAAGGCTCCTTC **p589S** 6FAM-CCATGCCCAGGAGGACTCGCCTTT-TAMRA	(0.1)	Trombley *et al.* (2010)
		GP	55	**F1129** (+) TCACCGCGAACCCAATG **R1183** (–) TCGCTTGTCATGGTTGGACTT **p1149S** 6FAM-ACCACCATTGCCC-MGBNFQ	34 (1.0)	Trombley *et al.* (2010)

Continued

Table 14.2. Continued.

Technique	Specificity	Gene target	Amplicon Size (bp)	Primers 5′ to 3′ [name, (polarity), sequence]	Sensitivity RNA copies or (PFU)/ reaction	Source
Q-RT-PCR	SUDV	VP40	80	**F5645** (+) CTATGGTTATCACCCAGGATTGTG **R5724** (–) GTAACTATCCTGCTTGTCCATGTG **p5674S** 6FAM-TGCCACTCTCCAGCCAGCCATCCG-TAMRA	(1.0)	Trombley *et al*. (2010)
		NP	73	**Ebo-SudBMG 1** (+) GCCATGGITTCAGGTTTGAG **EboSudBMG 1** (–) GGTIACATTGGGCAACAATTC A **Probe** FAM-ACGGTGCACATTCTCCTTTTCTCGGA-BHQ1	Sensitive but undetermined	Trombley *et al*. (2010)
		GP	77	**F583** (+) AGGATGGAGCTTTCTTCCTCTATG **R659** (–) TACCCCCTCAGCAAAATTGACT **p608SB** 6FAM-CAGGCTGGCTTCAACTGTAATTTACAGAGG-TAMRA	(0.1)	Towner *et al*. (2008)
		NP	80	**F1051** (+) CATGCAGAACAAGGGCTCATTC **R1130** (–) CTCATCAAACGGAAGATCACCATC **p1079S** 6FAM-CAACTTCCTGGCAAT-MGBNFQ	(0.1)	Trombley *et al*. (2010)
		NP	76	**Forward** (+) TGGAAAAAACATTAAGAGAACACTTGC **Reverse** (–) AGGAGAGAAACTGACCGGCAT **Probe** FAM-CATGCCGGAAGAGGAGACAACTGAAGC-BHQ1	3-38	Towner *et al*. (2007)
		NP	76	**F565** (+) 5′-TCTGACATGGATTACCACAAGATC **R640** (–) GGATGACTCTTTGCCGAACAATC **p597S** 6FAM-AGGTCTGTCCGTTCAA-MGBNFQ	(0.001)	Trombley *et al*. (2010)
		GP	80	**F2000** (+) TTTTCAATCCTCAACCGTAAGGC **R2079** (–) CAGTCCGGTCCCAGAATGTG **p2058A** 6FAM-CATGTGCCGCCCCATCGCTGC-TAMRA	584 (0.0001)	Trombley *et al*. (2010)

[a] In RNA copies/reaction as estimated by Towner *et al*. (2004) or related to this estimation.

cells cultures has been used to diagnose viral infection (Geisbert and Jahrling, 1990, 1995; Geisbert *et al.*, 1991, 1992). EM requires highly specialized equipment and trained personnel, which are only available at reference centres. The EM-based diagnosis, which has an intrinsically low sensitivity and is performed in hours, can be used to diagnose ebolavirus infection in the acute phase when viral titres are high.

14.5.6 Serology

Ebolavirus infection elicits IgM antibodies soon after infection, which can be detected for several months, and IgG antibodies that remain for several years. The detection of anti-EBOV IgM antibodies is indicative of a recent virus infection. In primates, fatal cases develop minimal or no antibody response, and the absence of antibodies cannot be used to rule out ebolavirus infection (Baize *et al.*, 1999; Ksiazek *et al.*, 1999a). Different tests have been developed to detect anti-ebolavirus antibodies.

Indirect immunofluorescence assays (IFAs) based on the binding of antibodies to ebolavirus-infected cells were developed early but lacked specificity (Van der Waals *et al.*, 1986; Ksiazek *et al.*, 1999b and references therein). IFAs with increased specificity were developed using HeLa cells expressing recombinant NP (Saijo *et al.*, 2001; Ikegami *et al.*, 2002).

Sensitive and specific ELISAs based on the binding of antibodies to gamma-irradiated ebolavirus antigens produced in infected cells were developed to detect anti-ebolavirus IgM in a capture test and IgG in a direct ELISA (Ksiazek *et al.*, 1999b). These tests have been extensively used to diagnose ebolavirus infection in reference and field laboratories. The cross-reaction of this ELISA with marburgvirus antibodies is limited (Gonzalez *et al.*, 2000). Cross-reactivity between ebolavirus species is low in the IgM capture ELISA but is strong in the IgG ELISA (Macneil *et al.*, 2011). ELISA tests using recombinant Ebolavirus GP, NP and VP35 proteins for the detection of antibodies have been developed (Prehaud *et al.*, 1998; Groen *et al.*, 2003; Ikegami *et al.*, 2003b; Niikura *et al.*, 2003; Nakayama *et al.*, 2010), but there are limited data on outbreak settings using these tests. ELISAs based on soluble forms of trimeric EBOV GP fused to the Fc fragments of human IgG1, which resemble GP expressed at the virus and cell surface, are likely to detect conformational epitopes not present in ELISAs based on fragments and monomeric forms of GP (Konduru *et al.*, 2011). Assays based on recombinant proteins limit the use of ebolavirus infectious materials and could be used for species-specific diagnosis based on proteins that display limited cross-reactivity (Nakayama *et al.*, 2010).

ELISAs based on viral particles, such as irradiated ebolavirus or replication-competent vesicular stomatitis virus (VSV) containing the ebolavirus GP, allow the evaluation of total anti-ebolavirus GP antibodies capable of binding to viral particles (Konduru *et al.*, 2011). These virus particle ELISAs detect anti-GP antibodies directed to epitopes on the trimeric GP displayed on a lipid envelope, which might be relevant to the protective effect of the antibody response.

Several other antibody assays have been used to diagnose ebolavirus infection. For instance, competition assays using a human monoclonal antibody raised against a highly conserved epitope in NP have been used to detect seroconversion from most species of ebolavirus (Meissner *et al.*, 2002). Western blot analyses using extracts of ebolavirus-infected cells or recombinant proteins have also been used to detect anti-ebolavirus antibodies.

14.5.7 Neutralization assays

The detection of neutralizing antibodies is commonly used to diagnose viral infections. In humans, low levels of anti-ebolavirus neutralizing antibodies appear late in the convalescence period (Peters *et al.*, 1996; Maruyama *et al.*, 1999). There are indications that neutralizing antibodies play a role

in protection against ebolavirus infection. The transfusion of convalescent-phase blood to infected patients in the 1995 Kikwit ebolavirus outbreak might have increased patient survival (Mupapa *et al.*, 1999; Sadek *et al.*, 1999). Polyclonal and monoclonal neutralizing antibodies protect mice, guinea pigs and NHPs against fatal challenge with ebolavirus (Dye *et al.*, 2012; Olinger *et al.*, 2012; Qiu *et al.*, 2012 and references therein). Due to the late onset and low levels of neutralizing antibodies in ebolavirus infection, neutralization tests have limited diagnostic value during early infection but provide useful information later in infection. Ebolavirus plaque assays have been developed (Moe *et al.*, 1981; Shurtleff *et al.*, 2012), and plaque reduction neutralization tests (PRNTs) (Jahrling, 1995) have been used to evaluate neutralizing antibodies in clinical and experimental samples under BSL-4 conditions. PRNT is laborious, requires more than 1 week for completion, and cannot be used with ebolavirus strains that induce limited or no cytopathic effect. The availability of the ebolavirus reverse genetics system (Volchkov *et al.*, 2001; Neumann *et al.*, 2002) allowed the introduction of the green fluorescent protein gene into the ebolavirus genome (Towner *et al.*, 2005), which has been used to develop fast neutralization tests based on the reduction of fluorescence in response to treatment with sera containing neutralizing antibodies (Kobinger *et al.*, 2011; Wong *et al.*, 2012 and references therein).

The use of reverse genetics systems to produce pseudotyped viruses containing the ebolavirus GP on the viral membrane of a wide variety of viruses, including retroviruses, rhabdoviruses and paramyxoviruses, facilitated the study of ebolavirus cell entry, immune responses and neutralization under BSL-2 conditions (Takada *et al.*, 1997; Wool-Lewis and Bates, 1998; Kobinger *et al.*, 2001; Garbutt *et al.*, 2004; Bukreyev *et al.*, 2006; Blaney *et al.*, 2011 and references therein). Neutralization assays based on replication-competent VSV pseudotypes containing the ebolavirus GP replacing the VSV-G envelope glycoprotein provide a useful alternative to evaluate anti-ebolavirus neutralizing antibodies under BSL-2 conditions (Bukreyev *et al.*, 2010; DiNapoli *et al.*, 2010; Konduru *et al.*, 2011; Ou *et al.*, 2011).

14.6 Differential Diagnoses

The natural infection of primates, pigs, dogs and bats, and perhaps duikers and some rodents, with ebolavirus (review in Olson *et al.*, 2012) results in a wide variety of outcomes, ranging from asymptomatic infections to high morbidity and mortality, with symptoms common to many other diseases. The diagnosis of infection in one duiker carcass out of six suggests that these animals might also be naturally infected with ebolavirus (Wittmann *et al.*, 2007), but there are no studies describing the disease in these animals. Ebolavirus infection cannot be differentially diagnosed based on clinical symptoms, which are common to many other infections. In primates, ebolavirus infections induce early non-specific symptoms that include severe acute fever. A loss of lymphocytes due to bystander apoptosis (Baize *et al.*, 1999) and a strong pro-inflammatory response (Villinger *et al.*, 1999) is also characteristic of ebolavirus infection. Coagulation disorders in later stages of the disease (Geisbert *et al.*, 2003b) are also induced by other haemorrhagic fever viruses and cannot be used for the differential diagnosis of ebolavirus infection. Primates develop a maculopapular rash on the trunk and limbs, bleeding from the gastrointestinal and urogenital tracts, and petechiae (review in Warfield *et al.*, 2009).

The subclinical disease in pigs experimentally infected with RESTV (Marsh *et al.*, 2011) and the severe respiratory syndrome in pigs infected with EBOV (Kobinger *et al.*, 2011) could be mistaken for other porcine respiratory diseases and cannot be used to differentially diagnose ebolavirus infection.

The sampling of dogs in outbreak areas revealed a high prevalence of ebolavirus IgG antibodies (Allela *et al.*, 2005). The ebolavirus antigen and nucleic acids were

not detected in dogs naturally exposed to ebolavirus, suggesting that these animals developed asymptomatic infections.

The presence of anti-ebolavirus antibodies in different species of fruit bats suggests that these animals could be a natural animal reservoir for EBOV (review in Olson *et al.*, 2012). These findings are also consistent with the asymptomatic disease observed in fruit and possibly insectivorous bats experimentally infected with EBOV (Swanepoel *et al.*, 1996). The long-term survival of infected bats might also pose a significant risk for the spread of EBOV to humans and other animals (Hayman *et al.*, 2010).

14.7 Treatment and Prevention

There is currently no FDA- or EU-approved treatment for filovirus infection. Controls of outbreaks are primarily achieved through quarantine, in which diagnosis, case management and preventative measures, such as universal precautions and barrier nursing procedures, play a significant role. When properly identified, transmission can be limited through patient isolation and case contact medical surveillance. Supportive care of patients is the medical objective, which includes oral fluid rehydration, palliative medications, nutritional supplementation and psychosocial support (Jeffs *et al.*, 2007; Roddy *et al.*, 2011).

During the past two decades, the development of medical countermeasures for filoviruses has received considerable attention, and vaccines and therapeutic options are now on the horizon. Prophylactic and post-exposure (similar to rabies vaccination after exposure) vaccinations are effective in animal models of disease and have been used in patients after laboratory exposure. Vaccination has long been the primary strategy to protect against infection. A variety of vaccines have been developed, including subunit vaccines, virus-like particles, vectored systems, DNA vaccines and live-attenuated virus systems that express the ebolavirus or marburgvirus proteins (Falzarano *et al.*, 2011). Vaccination with GP is sufficient to provide protection in animal models of disease but provides limited cross-protection and requires multiple virus proteins for protection against the various ebolavirus species.

Therapeutic interventions are being evaluated to treat infected patients. A variety of novel and repurposed small molecules and antibodies directed against the virus, critical host proteins or pathways associated with viral pathogenesis have been successfully used in experimental settings. Silencing RNA technologies and hyperimmune sera are the most advanced candidate treatments (Kudoyarova-Zubavichene *et al.*, 1999; Bausch *et al.*, 2007; Aman *et al.*, 2009; Geisbert *et al.*, 2010 and references therein). Similarly, the passive immunity conferred through the transfer of sera or monoclonal antibodies, long considered as potential interventions for Filovirus infections, has recently been shown as highly effective (Dye *et al.*, 2012; Olinger *et al.*, 2012; Qiu *et al.*, 2012). More recently, monoclonal antibody mixtures have been used to successfully treat ebolavirus-infected animals (Qiu *et al.*, 2012). Because zoonotic transmission precedes sporadic Filovirus human outbreaks, the use of veterinary vaccines and therapies could generate a barrier to disease in humans and could protect wild animals (Wong and Kobinger, 2012).

14.8 Conclusions

The widely varied outcomes of ebolavirus infection in different animals complicate diagnosis, which should be tailored to each animal species under study. The availability of assays that can be used under BSL-2 conditions has simplified the diagnosis of ebolavirus infection. The development of pseudotyped viruses containing GP and the availability of ebolavirus recombinant antigens allows the assessment of humoral and cellular immune responses under BSL-2 conditions. However, virus isolation, the

gold standard for the diagnosis of ebolavirus infection, can only be performed in BSL-4 facilities in reference laboratories using properly shipped samples. The validation of standardized BSL-2 ebolavirus diagnostic tests is now possible and highly desirable to facilitate the detection and control of future outbreaks of a zoonotic pathogen that is becoming more prevalent.

Disclaimer

The opinions and views expressed are of the authors and not of the US Government. The findings and conclusions in this article have not been formally disseminated by the FDA and should not be construed to represent any FDA determination or policy.

References

Allela, L., Boury, O., Pouillot, R., Delicat, A., Yaba, P., Kumulungui, B., Rouquet, P., Gonzalez, J.P. and Leroy, E.M. (2005) Ebola virus antibody prevalence in dogs and human risk. *Emerging Infectious Diseases* 11, 385–390.

Alvarez, C.P., Lasala, F., Carrillo, J., Muniz, O., Corbi, A.L. and Delgado, R. (2002) C-type lectins DC-SIGN and L-SIGN mediate cellular entry by Ebola virus in cis and in trans. *Journal of Virology* 76, 6841–6844.

Aman, M.J., Kinch, M.S., Warfield, K., Warren, T., Yunus, A., Enterlein, S., Stavale, E., Wang, P., Chang, S., Tang, Q., Porter, K., Goldblatt, M. and Bavari, S. (2009) Development of a broad-spectrum antiviral with activity against Ebola virus. *Antiviral Research* 83, 245–251.

Baize, S., Leroy, E.M., Georges-Courbot, M.C., Capron, M., Lansoud-Soukate, J., Debre, P., Fisher-Hoch, S.P., McCormick, J.B. and Georges, A.J. (1999) Defective humoral responses and extensive intravascular apoptosis are associated with fatal outcome in Ebola virus-infected patients. *Nature Medicine* 5, 423–426.

Bamberg, S., Kolesnikova, L., Moller, P., Klenk, H.D. and Becker, S. (2005) VP24 of Marburg virus influences formation of infectious particles. *Journal of Virology* 79, 13,421–13,433.

Barrette, R.W., Metwally, S.A., Rowland, J.M., Xu, L., Zaki, S.R., Nichol, S.T., Rollin, P.E., Towner, J.S., Shieh, W.J., Batten, B., Sealy, T.K., Carrillo, C., Moran, K.E., Bracht, A.J., Mayr, G.A., Sirios-Cruz, M., Catbagan, D.P., Lautner, E.A., Ksiazek, T.G., White, W.R. and McIntosh, M.T. (2009) Discovery of swine as a host for the Reston ebolavirus. *Science* 325, 204–206.

Basler, C.F. and Amarasinghe, G.K. (2009) Evasion of interferon responses by Ebola and Marburg viruses. *Journal of Interferon & Cytokine Research* 29, 511–520.

Bausch, D.G., Feldmann, H., Geisbert, T.W., Bray, M., Sprecher, A.G., Boumandouki, P., Rollin, P.E. and Roth, C. (2007) Outbreaks of filovirus hemorrhagic fever: time to refocus on the patient. *Journal of Infectious Diseases* 196, S136–141.

Bavari, S., Bosio, C.M., Wiegand, E., Ruthel, G., Will, A.B., Geisbert, T.W., Hevey, M., Schmaljohn, C., Schmaljohn, A. and Aman, M.J. (2002) Lipid raft microdomains: a gateway for compartmentalized trafficking of Ebola and Marburg viruses. *Journal of Experimental Medicine* 195, 593–602.

Becker, S., Rinne, C., Hofsass, U., Klenk, H.D. and Muhlberger, E. (1998) Interactions of Marburg virus nucleocapsid proteins. *Virology* 249, 406–417.

Blaney, J.E., Wirblich, C., Papaneri, A.B., Johnson, R.F., Myers, C.J., Juelich, T.L., Holbrook, M.R., Freiberg, A.N., Bernbaum, J.G., Jahrling, P.B., Paragas, J. and Schnell, M.J. (2011) Inactivated or live-attenuated bivalent vaccines that confer protection against rabies and Ebola viruses. *Journal of Virology* 85, 10,605–10,616.

Borio, L., Inglesby, T., Peters, C.J., Schmaljohn, A.L., Hughes, J.M., Jahrling, P.B., Ksiazek, T., Johnson, K.M., Meyerhoff, A., O'Toole, T., Ascher, M.S., Bartlett, J., Breman, J.G., Eitzen, E.M. Jr, Hamburg, M., Hauer, J., Henderson, D.A., Johnson, R.T., Kwik, G., Layton, M., Lillibridge, S., Nabel, G.J., Osterholm, M.T., Perl, T.M., Russell, P., Tonat, K. and Working Group on Civilian, B. (2002) Hemorrhagic fever viruses as biological weapons: medical and public health management. *JAMA* 287, 2391–2405.

Bradfute, S.B., Dye, J.M. Jr and Bavari, S. (2011) Filovirus vaccines. *Human Vaccines* 7, 701–711.

Bukreyev, A., Yang, L., Zaki, S.R., Shieh, W.J., Rollin, P.E., Murphy, B.R., Collins, P.L. and Sanchez, A. (2006) A single intranasal inoculation with a paramyxovirus-vectored vaccine protects guinea pigs against a lethal-dose Ebola virus challenge. *Journal of Virology* 80, 2267–2279.

Bukreyev, A.A., Dinapoli, J.M., Yang, L., Murphy, B.R. and Collins, P.L. (2010) Mucosal parainfluenza virus-vectored vaccine against Ebola virus replicates in the respiratory tract of vector-immune monkeys and is immunogenic. *Virology* 399, 290–298.

Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* 162, 156–159.

Dedkova, L.M., Kudoiarova, N.M., Chepurpov, A.A. and Ofitserov, V.I. (1994) Composition and immunochemical properties of goat immunoglobulins against the Ebola virus. *Voprosy Virusologii* 39, 229–232.

DiNapoli, J.M., Yang, L., Samal, S.K., Murphy, B.R., Collins, P.L. and Bukreyev, A. (2010) Respiratory tract immunization of non-human primates with a Newcastle disease virus-vectored vaccine candidate against Ebola virus elicits a neutralizing antibody response. *Vaccine* 29, 17–25.

Drosten, C., Gottig, S., Schilling, S., Asper, M., Panning, M., Schmitz, H. and Gunther, S. (2002) Rapid detection and quantification of RNA of Ebola and Marburg viruses, Lassa virus, Crimean-Congo hemorrhagic fever virus, Rift Valley fever virus, dengue virus and yellow fever virus by real-time reverse transcription-PCR. *Journal of Clinical Microbiology* 40, 2323–2330.

Dye, J.M., Herbert, A.S., Kuehne, A.I., Barth, J.F., Muhammad, M.A., Zak, S.E., Ortiz, R.A., Prugar, L.I. and Pratt, W.D. (2012) Postexposure antibody prophylaxis protects nonhuman primates from filovirus disease. *Proceedings of the National Academy of Sciences of the USA* 109, 5034–5039.

Egbring, R., Slenczka, W. and Baltzer, G. (1971) Clinical manifestations and mechanism of the haemorrhagic diathesis in Marburg virus disease. In: Martini, G.A. and Siegert, R. (eds) *Marburg Virus Disease*. Springer, New York, pp. 41–49.

Falzarano, D., Geisbert, T.W. and Feldmann, H. (2011) Progress in filovirus vaccine development: evaluating the potential for clinical use. *Expert Review of Vaccines* 10, 63–77.

Feldmann, H. and Kiley, M.P. (1999) Classification, structure and replication of filoviruses. *Current Topics in Microbiology and Immunology* 235, 1–21.

Feldmann, H., Muhlberger, E., Randolf, A., Will, C., Kiley, M.P., Sanchez, A. and Klenk, H.D. (1992) Marburg virus, a filovirus: messenger RNAs, gene order and regulatory elements of the replication cycle. *Virus Research* 24, 1–19.

Feldmann, H., Geisbert, T.W., Jahrling, P.B., Klenk, H.D., Netesov, S.V., Peter, C.J., Sanchez, A., Swanepoel, R. and Volchkov, V.E. (2005) Family filoviridae. In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U. and Ball, L.A. (eds) *Virus Taxonomy: VIIIth Report of the International Committee on Taxonomy of Viruses*. Elsevier/Academic Press, London, pp. 645–653.

Garbutt, M., Liebscher, R., Wahl-Jensen, V., Jones, S., Moller, P., Wagner, R., Volchkov, V., Klenk, H.D., Feldmann, H. and Stroher, U. (2004) Properties of replication-competent vesicular stomatitis virus vectors expressing glycoproteins of filoviruses and arenaviruses. *Journal of Virology* 78, 5458–5465.

Gear, J.H. (1989) Clinical aspects of African viral hemorrhagic fevers. *Reviews of Infectious Diseases* 11, S777–S782.

Gear, J.S., Cassel, G.A., Gear, A.J., Trappler, B., Clausen, L., Meyers, A.M., Kew, M.C., Bothwell, T.H., Sher, R., Miller, G.B., Schneider, J., Koornhof, H.J., Gomperts, E.D., Isaacson, M. and Gear, J.H. (1975) Outbreak of Marburg virus disease in Johannesburg. *British Medical Journal* 4, 489–493.

Geisbert, T.W. and Jahrling, P.B. (1990) Use of immunoelectron microscopy to show Ebola virus during the 1989 United States epizootic. *Journal of Clinical Pathology* 43, 813–816.

Geisbert, T.W. and Jahrling, P.B. (1995) Differentiation of filoviruses by electron microscopy. *Virus Research* 39, 129–150.

Geisbert, T.W., Rhoderick, J.B. and Jahrling, P.B. (1991) Rapid identification of Ebola virus and related filoviruses in fluid specimens using indirect immunoelectron microscopy. *Journal of Clinical Pathology* 44, 521–522.

Geisbert, T.W., Jahrling, P.B., Hanes, M.A. and Zack, P.M. (1992) Association of Ebola-related Reston virus particles and antigen with tissue lesions of monkeys imported to the United States. *Journal of Comparative Pathology* 106, 137–152.

Geisbert, T.W., Hensley, L.E., Larsen, T., Young, H.A., Reed, D.S., Geisbert, J.B., Scott, D.P., Kagan, E., Jahrling, P.B. and Davis, K.J. (2003a) Pathogenesis of Ebola hemorrhagic fever in cynomolgus macaques: evidence that dendritic cells are early and sustained targets of infection. *American Journal of Pathology* 163, 2347–2370.

Geisbert, T.W., Young, H.A., Jahrling, P.B., Davis, K.J., Kagan, E. and Hensley, L.E. (2003b) Mechanisms underlying coagulation abnormalities in Ebola hemorrhagic fever: overexpression of tissue factor in primate monocytes/macrophages is a key event. *Journal of Infectious Diseases* 188, 1618–1629.

Geisbert, T.W., Young, H.A., Jahrling, P.B., Davis, K.J., Larsen, T., Kagan, E. and Hensley, L.E. (2003c) Pathogenesis of Ebola hemorrhagic fever in primate models: evidence that hemorrhage is not a direct effect of virus-induced cytolysis of endothelial cells. *American Journal of Pathology* 163, 2371–2382.

Geisbert, T.W., Lee, A.C., Robbins, M., Geisbert, J.B., Honko, A.N., Sood, V., Johnson, J.C., de Jong, S., Tavakoli, I., Judge, A., Hensley, L.E. and MacLachlan, I. (2010) Postexposure protection of non-human primates against a lethal Ebola virus challenge with RNA interference: a proof-of-concept study. *Lancet* 375, 1896–1905.

Gonzalez, J.P., Nakoune, E., Slenczka, W., Vidal, P. and Morvan, J.M. (2000) Ebola and Marburg virus antibody prevalence in selected populations of the Central African Republic. *Microbes and Infection* 2, 39–44.

Goodchild, S.A., Dooley, H., Schoepp, R.J., Flajnik, M. and Lonsdale, S.G. (2011) Isolation and characterisation of Ebolavirus-specific recombinant antibody fragments from murine and shark immune libraries. *Molecular Immunology* 48, 2027–2037.

Groen, J., van den Hoogen, B.G., Burghoorn-Maas, C.P., Fooks, A.R., Burton, J., Clegg, C.J., Zeller, H. and Osterhaus, A.D. (2003) Serological reactivity of baculovirus-expressed Ebola virus VP35 and nucleoproteins. *Microbes and Infection* 5, 379–385.

Han, Z., Boshra, H., Sunyer, J.O., Zwiers, S.H., Paragas, J. and Harty, R.N. (2003) Biochemical and functional characterization of the Ebola virus VP24 protein: implications for a role in virus assembly and budding. *Journal of Virology* 77, 1793–1800.

Hayes, C.G., Burans, J.P., Ksiazek, T.G., Del Rosario, R.A., Miranda, M.E., Manaloto, C.R., Barrientos, A.B., Robles, C.G., Dayrit, M.M. and Peters, C.J. (1992) Outbreak of fatal illness among captive macaques in the Philippines caused by an Ebola-related filovirus. *American Journal of Tropical Medicine and Hygiene* 46, 664–671.

Hayman, D.T., Emmerich, P., Yu, M., Wang, L.F., Suu-Ire, R., Fooks, A.R., Cunningham, A.A. and Wood, J.L. (2010) Long-term survival of an urban fruit bat seropositive for Ebola and Lagos bat viruses. *PLoS One* 5, e11978.

Ikegami, T., Saijo, M., Niikura, M., Miranda, M.E., Calaor, A.B., Hernandez, M., Manalo, D.L., Kurane, I., Yoshikawa, Y. and Morikawa, S. (2002) Development of an immunofluorescence method for the detection of antibodies to Ebola virus subtype Reston by the use of recombinant nucleoprotein-expressing HeLa cells. *Microbiology and Immunology* 46, 633–638.

Ikegami, T., Niikura, M., Saijo, M., Miranda, M.E., Calaor, A.B., Hernandez, M., Acosta, L.P., Manalo, D.L., Kurane, I., Yoshikawa, Y. and Morikawa, S. (2003a) Antigen capture enzyme-linked immunosorbent assay for specific detection of Reston Ebola virus nucleoprotein. *Clinical and Diagnostic Laboratory Immunology* 10, 552–557.

Ikegami, T., Saijo, M., Niikura, M., Miranda, M.E., Calaor, A.B., Hernandez, M., Manalo, D.L., Kurane, I., Yoshikawa, Y. and Morikawa, S. (2003b) Immunoglobulin G enzyme-linked immunosorbent assay using truncated nucleoproteins of Reston Ebola virus. *Epidemiology and Infection* 130, 533–539.

Jahrling, P.B. (1995) Filoviruses and arenaviruses. In: Murray, P.R., Baron, E.J., Pfaller, M.A., Tenover, F.C. and Yolken, R.H. (eds) *Manual of Clinical Microbiology*. ASM Press, Washington, DC, pp. 1068–1081.

Jahrling, P.B., Geisbert, T.W., Dalgard, D.W., Johnson, E.D., Ksiazek, T.G., Hall, W.C. and Peters, C.J. (1990) Preliminary report: isolation of Ebola virus from monkeys imported to USA. *Lancet* 335, 502–505.

Jeffers, S.A., Sanders, D.A. and Sanchez, A. (2002) Covalent modifications of the Ebola virus glycoprotein. *Journal of Virology* 76, 12,463–12,472.

Jeffs, B., Roddy, P., Weatherill, D., de la Rosa, O., Dorion, C., Iscla, M., Grovas, I., Palma, P.P., Villa, L., Bernal, O., Rodriguez-Martinez, J., Barcelo, B., Pou, D. and Borchert, M. (2007) The Medecins Sans Frontieres intervention in the Marburg hemorrhagic fever epidemic, Uige, Angola, 2005. I. Lessons learned in the hospital. *Journal of Infectious Diseases* 196, S154–161.

Kobinger, G.P., Weiner, D.J., Yu, Q.C. and Wilson, J.M. (2001) Filovirus-pseudotyped lentiviral vector can efficiently and stably transduce airway epithelia in vivo. *Nature Biotechnology* 19, 225–230.

Kobinger, G.P., Leung, A., Neufeld, J., Richardson, J.S., Falzarano, D., Smith, G., Tierney, K., Patel, A. and Weingartl, H.M. (2011) Replication, pathogenicity, shedding and transmission of Zaire ebolavirus in pigs. *Journal of Infectious Diseases* 204, 200–208.

Kondratowicz, A.S., Lennemann, N.J., Sinn, P.L., Davey, R.A., Hunt, C.L., Moller-Tank, S., Meyerholz, D.K., Rennert, P., Mullins, R.F., Brindley, M., Sandersfeld, L.M., Quinn, K., Weller, M., McCray, P.B. Jr, Chiorini, J. and Maury, W. (2011) T-cell immunoglobulin and mucin domain 1 (TIM-1) is a receptor for Zaire Ebolavirus and Lake Victoria Marburgvirus. *Proceedings of the National Academy of Sciences of the USA* 108, 8426–8431.

Konduru, K., Bradfute, S.B., Jacques, J., Manangeeswaran, M., Nakamura, S., Morshed, S., Wood, S.C., Bavari, S. and Kaplan, G.G. (2011) Ebola virus glycoprotein Fc fusion protein confers protection against lethal challenge in vaccinated mice. *Vaccine* 29, 2968–2977.

Krasnianskii, V.P., Mikhaĭlov, V.V., Borisevich, I.V., Gradoboev, V.N., Evseev, A.A. and Pshenichnov, V.A. (1994) The isolation of hyperimmune horse serum to the Ebola virus. *Voprosy Virusologii* 39, 91–92.

Ksiazek, T.G., Rollin, P.E., Jahrling, P.B., Johnson, E., Dalgard, D.W. and Peters, C.J. (1992) Enzyme immunosorbent assay for Ebola virus antigens in tissues of infected primates. *Journal of Clinical Microbiology* 30, 947–950.

Ksiazek, T.G., Rollin, P.E., Williams, A.J., Bressler, D.S., Martin, M.L., Swanepoel, R., Burt, F.J., Leman, P.A., Khan, A.S., Rowe, A.K., Mukunu, R., Sanchez, A. and Peters, C.J. (1999a) Clinical virology of Ebola hemorrhagic fever (EHF): virus, virus antigen and IgG and IgM antibody findings among EHF patients in Kikwit, Democratic Republic of the Congo, 1995. *Journal of Infectious Diseases* 179, S177–187.

Ksiazek, T.G., West, C.P., Rollin, P.E., Jahrling, P.B. and Peters, C.J. (1999b) ELISA for the detection of antibodies to Ebola viruses. *Journal of Infectious Diseases* 179, S192–198.

Kudoyarova-Zubavichene, N.M., Sergeyev, N.N., Chepurnov, A.A. and Netesov, S.V. (1999) Preparation and use of hyperimmune serum for prophylaxis and therapy of Ebola virus infections. *Journal of Infectious Diseases* 179, S218–223.

Kuhn, J.H. (2008) Filoviruses. A compendium of 40 years of epidemiological, clinical and laboratory studies. *Archives of Virology Supplement* 20, 13–360.

Kuhn, J.H., Becker, S., Ebihara, H., Geisbert, T.W., Johnson, K.M., Kawaoka, Y., Lipkin, W.I., Negredo, A.I., Netesov, S.V., Nichol, S.T., Palacios, G., Peters, C.J., Tenorio, A., Volchkov, V.E. and Jahrling, P.B. (2010) Proposal for a revised taxonomy of the family Filoviridae: classification, names of taxa and viruses and virus abbreviations. *Archives of Virology* 155, 2083–2103.

Kuhn, J.H., Bao, Y., Bavari, S., Becker, S., Bradfute, S., Brister, J.R., Bukreyev, A.A., Chandran, K., Davey, R.A., Dolnik, O., Dye, J.M., Enterlein, S., Hensley, L.E., Honko, A.N., Jahrling, P.B., Johnson, K.M., Kobinger, G., Leroy, E.M., Lever, M.S., Muhlberger, E., Netesov, S.V., Olinger, G.G., Palacios, G., Patterson, J.L., Paweska, J.T., Pitt, L., Radoshitzky, S.R., Saphire, E.O., Smither, S.J., Swanepoel, R., Towner, J.S., van der Groen, G., Volchkov, V.E., Wahl-Jensen, V., Warren, T.K., Weidmann, M. and Nichol, S.T. (2012) Virus nomenclature below the species level: a standardized nomenclature for natural variants of viruses assigned to the family Filoviridae. *Archives of Virology* 158, 301–311.

Kurosaki, Y. and Yasuda, J. (2011) Ebola viruses. In: Lui, D. (ed.) *Molecular Detection of Human Viral Pathogens*. CRC Press, Boca Raton, Florida, pp. 559–572.

Larsen, T., Stevens, E.L., Davis, K.J., Geisbert, J.B., Daddario-DiCaprio, K.M., Jahrling, P.B., Hensley, L.E. and Geisbert, T.W. (2007) Pathologic findings associated with delayed death in nonhuman primates experimentally infected with Zaire Ebola virus. *Journal of Infectious Diseases* 196, S323–328.

Le Guenno, B., Formenty, P., Wyers, M., Gounon, P., Walker, F. and Boesch, C. (1995) Isolation and partial characterisation of a new strain of Ebola virus. *Lancet* 345, 1271–1274.

Leroy, E.M., Baize, S., Lu, C.Y., McCormick, J.B., Georges, A.J., Georges-Courbot, M.C., Lansoud-Soukate, J. and Fisher-Hoch, S.P. (2000) Diagnosis of Ebola haemorrhagic fever by RT-PCR in an epidemic setting. *Journal of Medical Virology* 60, 463–467.

Leroy, E.M., Rouquet, P., Formenty, P., Souquiere, S., Kilbourne, A., Froment, J.M., Bermejo, M., Smit, S., Karesh, W., Swanepoel, R., Zaki, S.R. and Rollin, P.E. (2004) Multiple Ebola virus transmission events and rapid decline of central African wildlife. *Science* 303, 387–390.

Lucht, A., Grunow, R., Moller, P., Feldmann, H. and Becker, S. (2003) Development, characterization and use of monoclonal VP40-antibodies for the detection of Ebola virus. *Journal of Virological Methods* 111, 21–28.

Lucht, A., Grunow, R., Otterbein, C., Moller, P., Feldmann, H. and Becker, S. (2004) Production of monoclonal antibodies and development of an antigen capture ELISA directed against the envelope glycoprotein GP of Ebola virus. *Medical Microbiology and Immunology* 193, 181–187.

Macneil, A., Reed, Z. and Rollin, P.E. (2011) Serologic cross-reactivity of human IgM and IgG antibodies to five species of Ebola virus. *PLoS Neglected Tropical Diseases 5*, e1175.

Mahanty, S. and Bray, M. (2004) Pathogenesis of filoviral haemorrhagic fevers. *Lancet Infectious Diseases* 4, 487–498.

Marsh, G.A., Haining, J., Robinson, R., Foord, A., Yamada, M., Barr, J.A., Payne, J., White, J., Yu, M., Bingham, J., Rollin, P.E., Nichol, S.T., Wang, L.F. and Middleton, D. (2011) Ebola Reston virus infection of pigs: clinical significance and transmission potential. *Journal of Infectious Diseases* 204, S804–809.

Martini, G.A. (1971) Marburg virus disease. Clinical syndrome. In: Martini, G.A. and Siegert, R. (eds) *Marburg Virus Disease*. Springer, New York, pp. 1–9.

Maruyama, T., Rodriguez, L.L., Jahrling, P.B., Sanchez, A., Khan, A.S., Nichol, S.T., Peters, C.J., Parren, P.W. and Burton, D.R. (1999) Ebola virus can be effectively neutralized by antibody produced in natural human infection. *Journal of Virology* 73, 6024–6030.

Mehedi, M., Falzarano, D., Seebach, J., Hu, X., Carpenter, M.S., Schnittler, H.J. and Feldmann, H. (2011) A new Ebola virus nonstructural glycoprotein expressed through RNA editing. *Journal of Virology* 85, 5406–5414.

Meissner, F., Maruyama, T., Frentsch, M., Hessell, A.J., Rodriguez, L.L., Geisbert, T.W., Jahrling, P.B., Burton, D.R. and Parren, P.W. (2002) Detection of antibodies against the four subtypes of Ebola virus in sera from any species using a novel antibody-phage indicator assay. *Virology* 300, 236–243.

Miranda, M.E. and Miranda, N.L. (2011) Reston ebolavirus in humans and animals in the Philippines: a review. *Journal of Infectious Diseases* 204, S757–760.

Moe, J.B., Lambert, R.D. and Lupton, H.W. (1981) Plaque assay for Ebola virus. *Journal of Clinical Microbiology* 13, 791–793.

Mohamadzadeh, M., Chen, L. and Schmaljohn, A.L. (2007) How Ebola and Marburg viruses battle the immune system. *Nature Reviews Immunology* 7, 556–567.

Morikawa, S., Saijo, M. and Kurane, I. (2007) Current knowledge on lower virulence of Reston Ebola virus [Connaissances actuelles sur la moindre virulence du virus Ebola Reston]. *Comparative Immunology, Microbiology and Infectious Diseases* 30, 391–398.

Mühlberger, E. (2007) Filovirus replication and transcription. *Future Virology* 2, 205–215.

Mühlberger, E., Lotfering, B., Klenk, H.D. and Becker, S. (1998) Three of the four nucleocapsid proteins of Marburg virus, NP, VP35 and L, are sufficient to mediate replication and transcription of Marburg virus-specific monocistronic minigenomes. *Journal of Virology* 72, 8756–8764.

Mühlberger, E., Weik, M., Volchkov, V.E., Klenk, H.D. and Becker, S. (1999) Comparison of the transcription and replication strategies of marburg virus and Ebola virus by using artificial replication systems. *Journal of Virology* 73, 2333–2342.

Mupapa, K., Massamba, M., Kibadi, K., Kuvula, K., Bwaka, A., Kipasa, M., Colebunders, R. and Muyembe-Tamfum, J.J. (1999) Treatment of Ebola hemorrhagic fever with blood transfusions from convalescent patients. International Scientific and Technical Committee. *Journal of Infectious Diseases* 179, S18–23.

Nakayama, E., Yokoyama, A., Miyamoto, H., Igarashi, M., Kishida, N., Matsuno, K., Marzi, A., Feldmann, H., Ito, K., Saijo, M. and Takada, A. (2010) Enzyme-linked immunosorbent assay for detection of filovirus species-specific antibodies. *Clinical Vaccine Immunology* 17, 1723–1728.

Negredo, A., Palacios, G., Vazquez-Moron, S., Gonzalez, F., Dopazo, H., Molero, F., Juste, J., Quetglas, J., Savji, N., de la Cruz Martinez, M., Herrera, J.E., Pizarro, M., Hutchison, S.K., Echevarria, J.E., Lipkin, W.I. and Tenorio, A. (2011) Discovery of an ebolavirus-like filovirus in Europe. *PLoS Pathogens* 7, e1002304.

Neumann, G., Feldmann, H., Watanabe, S., Lukashevich, I. and Kawaoka, Y. (2002) Reverse genetics demonstrates that proteolytic processing of the Ebola virus glycoprotein is not essential for replication in cell culture. *Journal of Virology* 76, 406–410.

Niikura, M., Ikegami, T., Saijo, M., Kurane, I., Miranda, M.E. and Morikawa, S. (2001) Detection of Ebola viral antigen by enzyme-linked immunosorbent assay using a novel monoclonal antibody to nucleoprotein. *Journal of Clinical Microbiology* 39, 3267–3271.

Niikura, M., Ikegami, T., Saijo, M., Kurata, T., Kurane, I. and Morikawa, S. (2003) Analysis of linear B-cell epitopes of the nucleoprotein of Ebola virus that distinguish Ebola virus subtypes. *Clinical and Diagnostic Laboratory Immunology* 10, 83–87.

Ogawa, H., Miyamoto, H., Ebihara, H., Ito, K., Morikawa, S., Feldmann, H. and Takada, A. (2011) Detection of all known filovirus species by reverse transcription-polymerase chain reaction using a primer set specific for the viral nucleoprotein gene. *Journal of Virological Methods* 171, 310–313.

Olinger, G.G. Jr, Pettitt, J., Kim, D., Working, C., Bohorov, O., Bratcher, B., Hiatt, E., Hume, S.D., Johnson, A.K., Morton, J., Pauly, M., Whaley, K.J., Lear, C.M., Biggins, J.E., Scully, C., Hensley, L. and Zeitlin, L. (2012) Delayed treatment of Ebola virus infection with plant-derived monoclonal antibodies provides protection in rhesus macaques. *Proceedings of the National Academy of Sciences of the USA* 109, 18,030–18,035.

Olson, S.H., Reed, P., Cameron, K.N., Ssebide, B.J., Johnson, C.K., Morse, S.S., Karesh, W.B., Mazet, J.A. and Joly, D.O. (2012) Dead or alive: animal sampling during Ebola hemorrhagic fever outbreaks in humans. *Emerging Health Threats Journal* 5, 10.3402/ehtj.v5i0.9134.

Ou, W., Delisle, J., Konduru, K., Bradfute, S., Radoshitzky, S.R., Retterer, C., Kota, K., Bavari, S., Kuhn, J.H., Jahrling, P.B., Kaplan, G. and Wilson, C.A. (2011) Development and characterization of rabbit and mouse antibodies against ebolavirus envelope glycoproteins. *Journal of Virological Methods* 174, 99–109.

Panning, M., Laue, T., Olschlager, S., Eickmann, M., Becker, S., Raith, S., Courbot, M.C., Nilsson, M., Gopal, R., Lundkvist, A., Caro, A., Brown, D., Meyer, H., Lloyd, G., Kummerer, B.M., Gunther, S. and Drosten, C. (2007) Diagnostic reverse-transcription polymerase chain reaction kit for filoviruses based on the strain collections of all European biosafety level 4 laboratories. *Journal of Infectious Diseases* 196, S199–204.

Peters, C.J., Sanchez, A., Rollin, P.E., Ksiazek, T.G. and Murphy, F.A. (1996) Marburg and Ebola viruses. In: Fields, B.N., Knipe, D.M. and Howley, P.M. (eds) *Fields Virology*. Raven Press, New York, pp. 1161–1176.

Prehaud, C., Hellebrand, E., Coudrier, D., Volchkov, V.E., Volchkova, V.A., Feldmann, H., Le Guenno, B. and Bouloy, M. (1998) Recombinant Ebola virus nucleoprotein and glycoprotein (Gabon 94 strain) provide new tools for the detection of human infections. *Journal of General Virology* 79, 2565–2572.

Qiu, X., Audet, J., Wong, G., Pillet, S., Bello, A., Cabral, T., Strong, J.E., Plummer, F., Corbett, C.R., Alimonti, J.B. and Kobinger, G.P. (2012) Successful treatment of Ebola virus-infected cynomolgus macaques with monoclonal antibodies. *Science Translational Medicine* 4, 138ra181.

Regnery, R.L., Johnson, K.M. and Kiley, M.P. (1980) Virion nucleic acid of Ebola virus. *Journal of Virology* 36, 465–469.

Richardson, J.S., Dekker, J.D., Croyle, M.A. and Kobinger, G.P. (2010) Recent advances in Ebolavirus vaccine development. *Human Vaccines* 6, 439–449.

Roddy, P., Colebunders, R., Jeffs, B., Palma, P.P., Van Herp, M. and Borchert, M. (2011) Filovirus hemorrhagic fever outbreak case management: a review of current and future treatment options. *Journal of Infectious Diseases* 204, S791–795.

Rodriguez, L.L., De Roo, A., Guimard, Y., Trappier, S.G., Sanchez, A., Bressler, D., Williams, A.J., Rowe, A.K., Bertolli, J., Khan, A.S., Ksiazek, T.G., Peters, C.J. and Nichol, S.T. (1999) Persistence and genetic stability of Ebola virus during the outbreak in Kikwit, Democratic Republic of the Congo, 1995. *Journal of Infectious Diseases* 179, S170–176.

Rouquet, P., Froment, J.M., Bermejo, M., Kilbourn, A., Karesh, W., Reed, P., Kumulungui, B., Yaba, P., Delicat, A., Rollin, P.E. and Leroy, E.M. (2005) Wild animal mortality monitoring and human Ebola outbreaks, Gabon and Republic of Congo, 2001–2003. *Emerging Infectious Diseases* 11, 283–290.

Ryabchikova, E., Kolesnikova, L., Smolina, M., Tkachev, V., Pereboeva, L., Baranova, S., Grazhdantseva, A. and Rassadkin, Y. (1996) Ebola virus infection in guinea pigs: presumable role of granulomatous inflammation in pathogenesis. *Archives of Virology* 141, 909–921.

Sadek, R.F., Khan, A.S., Stevens, G., Peters, C.J. and Ksiazek, T.G. (1999) Ebola hemorrhagic fever, Democratic Republic of the Congo, 1995: determinants of survival. *Journal of Infectious Diseases* 179, S24–27.

Saijo, M., Niikura, M., Morikawa, S. and Kurane, I. (2001) Immunofluorescence method for detection of Ebola virus immunoglobulin g, using HeLa cells which express recombinant nucleoprotein. *Journal of Clinical Microbiology* 39, 776–778.

Sanchez, A., Kiley, M.P., Holloway, B.P. and Auperin, D.D. (1993) Sequence analysis of the Ebola virus genome: organization, genetic elements and comparison with the genome of Marburg virus. *Virus Research* 29, 215–240.

Sanchez, A., Trappier, S.G., Mahy, B.W., Peters, C.J. and Nichol, S.T. (1996) The virion glycoproteins of Ebola viruses are encoded in two reading frames and are expressed through transcriptional editing. *Proceedings of the National Academy of Sciences of the USA* 93, 3602–3607.

Sanchez, A., Ksiazek, T.G., Rollin, P.E., Miranda, M.E., Trappier, S.G., Khan, A.S., Peters, C.J. and Nichol, S.T. (1999) Detection and molecular characterization of Ebola viruses causing disease in human and nonhuman primates. *Journal of Infectious Diseases* 179, S164–169.

Sanchez, A., Geisbert, T.W. and Feldmann, H. (2007) Filoviridae: Marburg and Ebola viruses. In: Knipe, D.M. and Howley, P.M. (eds) *Fields Virology*. Lippincott Williams & Wilkins, Philadelphia, Pennsylvania, pp. 1409–1448.

Shurtleff, A.C., Biggins, J.E., Keeney, A.E., Zumbrun, E.E., Bloomfield, H.A., Kuehne, A., Audet, J.L., Alfson, K.J., Griffiths, A., Olinger, G.G. and Bavari, S. (2012) Standardization of the filovirus plaque assay for use in preclinical studies. *Viruses* 4, 3511–3530.

Stephens, K.W., Hutchins, R.J. and Dauphin, L.A. (2010) Cross-platform evaluation of commercial real-time reverse transcription PCR master mix kits using a quantitative 5′ nuclease assay for Ebola virus. *Molecular and Cellular Probes* 24, 370–375.

Swanepoel, R., Leman, P.A., Burt, F.J., Zachariades, N.A., Braack, L.E., Ksiazek, T.G., Rollin, P.E., Zaki, S.R. and Peters, C.J. (1996) Experimental inoculation of plants and animals with Ebola virus. *Emerging Infectious Diseases* 2, 321–325.

Takada, A., Robison, C., Goto, H., Sanchez, A., Murti, K.G., Whitt, M.A. and Kawaoka, Y. (1997) A system for functional analysis of Ebola virus glycoprotein. *Proceedings of the National Academy of Sciences of the USA* 94, 14,764–14,769.

Towner, J.S., Rollin, P.E., Bausch, D.G., Sanchez, A., Crary, S.M., Vincent, M., Lee, W.F., Spiropoulou, C.F., Ksiazek, T.G., Lukwiya, M., Kaducu, F., Downing, R. and Nichol, S.T. (2004) Rapid diagnosis of Ebola hemorrhagic fever by reverse transcription-PCR in an outbreak setting and assessment of patient viral load as a predictor of outcome. *Journal of Virology* 78, 4330–4341.

Towner, J.S., Paragas, J., Dover, J.E., Gupta, M., Goldsmith, C.S., Huggins, J.W. and Nichol, S.T. (2005) Generation of eGFP expressing recombinant Zaire ebolavirus for analysis of early pathogenesis events and high-throughput antiviral drug screening. *Virology* 332, 20–27.

Towner, J.S., Sealy, T.K., Ksiazek, T.G. and Nichol, S.T. (2007) High-throughput molecular detection of hemorrhagic fever virus threats with applications for outbreak settings. *Journal of Infectious Diseases* 196, S205–212.

Towner, J.S., Sealy, T.K., Khristova, M.L., Albarino, C.G., Conlan, S., Reeder, S.A., Quan, P.L., Lipkin, W.I., Downing, R., Tappero, J.W., Okware, S., Lutwama, J., Bakamutumaho, B., Kayiwa, J., Comer, J.A., Rollin, P.E., Ksiazek, T.G. and Nichol, S.T. (2008) Newly discovered Ebola virus associated with hemorrhagic fever outbreak in Uganda. *PLoS Pathogens* 4, e1000212.

Trombley, A.R., Wachter, L., Garrison, J., Buckley-Beason, V.A., Jahrling, J., Hensley, L.E., Schoepp, R.J., Norwood, D.A., Goba, A., Fair, J.N. and Kulesh, D.A. (2010) Comprehensive panel of real-time TaqMan polymerase chain reaction assays for detection and absolute quantification of filoviruses, arenaviruses and New World hantaviruses. *American Journal of Tropical Medicine and Hygiene* 82, 954–960.

Van der Waals, F.W., Pomeroy, K.L., Goudsmit, J., Asher, D.M. and Gajdusek, D.C. (1986) Hemorrhagic fever virus infections in an isolated rainforest area of central Liberia. Limitations of the indirect immunofluorescence slide test for antibody screening in Africa. *Tropical and Geographical Medicine* 38, 209–214.

Villinger, F., Rollin, P.E., Brar, S.S., Chikkala, N.F., Winter, J., Sundstrom, J.B., Zaki, S.R., Swanepoel, R., Ansari, A.A. and Peters, C.J. (1999) Markedly elevated levels of interferon (IFN)-gamma, IFN-alpha, interleukin (IL)-2, IL-10 and tumor necrosis factor-alpha associated with fatal Ebola virus infection. *Journal of Infectious Diseases* 179, S188–191.

Volchkov, V.E., Becker, S., Volchkova, V.A., Ternovoj, V.A., Kotov, A.N., Netesov, S.V. and Klenk, H.D. (1995) GP mRNA of Ebola virus is edited by the Ebola virus polymerase and by T7 and vaccinia virus polymerases. *Virology* 214, 421–430.

Volchkov, V.E., Feldmann, H., Volchkova, V.A. and Klenk, H.D. (1998) Processing of the Ebola virus glycoprotein by the proprotein convertase furin. *Proceedings of the National Academy of Sciences of the USA* 95, 5762–5767.

Volchkov, V.E., Volchkova, V.A., Muhlberger, E., Kolesnikova, L.V., Weik, M., Dolnik, O. and Klenk, H.D. (2001) Recovery of infectious Ebola virus from complementary DNA: RNA editing of the GP gene and viral cytotoxicity. *Science* 291, 1965–1969.

Warfield, K.L., Deal, E.M. and Bavari, S. (2009) Filovirus infections. *Journal of the American Veterinary Medical Association* 234, 1130–1139.

Weingartl, H.M., Embury-Hyatt, C., Nfon, C., Leung, A., Smith, G. and Kobinger, G. (2012) Transmission of Ebola virus from pigs to non-human primates. *Scientific Reports* 2, 811.

White, J.M. and Schornberg, K.L. (2012) A new player in the puzzle of filovirus entry. *Nature Reviews Microbiology* 10, 317–322.

WHO (1978a) Ebola haemorrhagic fever in Sudan, 1976: Report of a WHO/International Study Team. *Bulletin of the World Health Organization* 56, 247–270.

WHO (1978b) Ebola haemorrhagic fever in Zaire, 1976: Report of an International Commission. *Bulletin of the World Health Organization* 56, 271–293.

WHO (2009) *WHO Experts Consultation on Ebola Reston Pathogenicity in Humans*. World Health Organization, Geneva.

Wittmann, T.J., Biek, R., Hassanin, A., Rouquet, P., Reed, P., Yaba, P., Pourrut, X., Real, L.A., Gonzalez, J.P. and Leroy, E.M. (2007) Isolates of Zaire ebolavirus from wild apes reveal genetic lineage and recombinants. *Proceedings of the National Academy of Sciences of the USA* 104, 17,123–17,127.

Wong, G. and Kobinger, G. (2012) A strategy to simultaneously eradicate the natural reservoirs of rabies and Ebola virus. *Expert Review in Vaccines* 11, 163–166.

Wong, G., Richardson, J.S., Pillet, S., Patel, A., Qiu, X., Alimonti, J., Hogan, J., Zhang, Y., Takada, A., Feldmann, H. and Kobinger, G.P. (2012) Immune parameters correlate with protection against Ebola virus infection in rodents and nonhuman primates. *Science Translational Medicine* 4, 158ra146.

Wool-Lewis, R.J. and Bates, P. (1998) Characterization of Ebola virus entry by using pseudotyped viruses: identification of receptor-deficient cell lines. *Journal of Virology* 72, 3155–3160.

Yu, J.S., Liao, H.X., Gerdon, A.E., Huffman, B., Scearce, R.M., McAdams, M., Alam, S.M., Popernack, P.M., Sullivan, N.J., Wright, D., Cliffel, D.E., Nabel, G.J. and Haynes, B.F. (2006) Detection of Ebola virus envelope using monoclonal and polyclonal antibodies in ELISA, surface plasmon resonance and a quartz crystal microbalance immunosensor. *Journal of Virological Methods* 137, 219–228.

Zaki, S.R., Shieh, W.J., Greer, P.W., Goldsmith, C.S., Ferebee, T., Katshitshi, J., Tshioko, F.K., Bwaka, M.A., Swanepoel, R., Calain, P., Khan, A.S., Lloyd, E., Rollin, P.E., Ksiazek, T.G. and Peters, C.J. (1999) A novel immunohistochemical assay for the detection of Ebola virus in skin: implications for diagnosis, spread and surveillance of Ebola hemorrhagic fever. Commission de Lutte contre les Epidemies á Kikwit. *Journal of Infectious Diseases* 179, S36–47.

15 *Marburg Marburgvirus*

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15.1 Introduction

Marburg virus (MARV), the aetiological agent of severe haemorrhagic fever in humans and non-human primates, was discovered in 1967 during an outbreak in Germany and former Yugoslavia after the importation of infected vervet monkeys (*Chlorocebus aethiops*) from Uganda, Eastern Africa (Smith *et al.*, 1967). MARV belongs to the family *Filoviridae* that together with the *Paramyxoviridae*, *Rhabdoviridae* and *Bornaviridae* constitute the order *Mononegavirales*, important pathogens for humans and animals (Lamb, 2007). The mononegavirads are characterized by having linear, negative-sense, single-stranded RNA genome and similar replicative strategies (Pringle, 1997; Lamb, 2007). *Marburgvirus* and *Ebolavirus* are the two main recognized genera of the *Filoviridae* family, although a recent report that revised the taxonomy of the family suggested the incorporation of a new genus, the *Cuevavirus* (Kuhn *et al.*, 2010). Marburgviruses are composed of several lineages that differ by less than 30% at the nucleotide level, thus the genus is represented by a single species, namely *Marburg marburgvirus*, with two virus members Marburg virus (MARV) and Ravn virus (RAVV) (Towner *et al.*, 2006; Kuhn *et al.*, 2010). The members of the ebolaviruses are divergent enough (i.e. ≥30%) to include at least five virus species: *Zaire ebolavirus*, with Ebola virus (EBOV) (World Health Organization (WHO), 1976; Kuhn *et al.*, 2010); *Sudan ebolavirus*, with Sudan virus (SUDV) (WHO, 1976; Kuhn *et al.*, 2010); *Reston ebolavirus*, with Reston virus (RESTV) (Jahrling *et al.*, 1990; Kuhn *et al.*, 2010); *Taï Forest ebolavirus*, with Taï Forest virus (TAFV) (Le Guenno *et al.*, 1995; Kuhn *et al.*, 2010); and *Bundibugyo ebolavirus*, with Bundibugyo virus (BDBV) (Towner *et al.*, 2006; Kuhn *et al.*, 2010). A novel filovirus species, tentatively called *Lloviu cuevavirus*, with Lloviu virus (LLOV), was accidentally identified from an insectivorous bat (*Miniopterus schreibersii*) die-off in northern Spain in 2002 and was assigned to the new genus *Cuevavirus* (Kuhn *et al.*, 2010; Negredo *et al.*, 2011).

The gene distribution of filoviruses follows the same basic genomic structure, starting at the 3′ end with the nucleoprotein (NP), followed by the phosphoprotein or VP35, major matrix protein or VP40, spike glycoprotein (GP), minor nucleoprotein or VP30, minor matrix protein or VP24, and the large protein (L) or the viral RNA-dependent RNA polymerase at its 5′ end (Sanchez *et al.*, 2007). The genome of marburgviruses differ from ebolaviruses by the presence of one gene overlap between VP30

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and VP24 genes, as opposed to two or three gene overlaps between VP35 and VP40, GP and VP30, and VP24 and L, respectively (Feldmann *et al.*, 1992; Sanchez *et al.*, 1993, 1996; Ikegami *et al.*, 2001). Further genomic difference was demonstrated for the GP gene: in marburgviruses the gene is transcribed as a full-length monocistronic mRNA, but in ebolaviruses the full-length transcript depends on the incorporation of a non-template adenosine at a site that resembles a putative transcriptional end signal, otherwise a short transcript that results in a secreted form of the protein (SGP) is the dominant form produced (Sanchez *et al.*, 1993, 1996). Functional studies of filovirus replication showed that the nucleocapsid complex of marburgviruses requires only three proteins, NP, VP35 and L for transcription and replication, whereas the ebolaviruses require the addition of VP30 for transcription activation (Mühlberger *et al.*, 1998, 1999). This last information was experimentally verified with artificial replication systems developed for both filoviruses.

15.2 Marburgvirus Infection

15.2.1 Epidemiology

Marburgvirus and ebolavirus infection cause severe haemorrhagic fever and are among the most virulent pathogens that affect human and non-human primates, and possibly swine (Mahanty and Bray, 2004; Barrette *et al.*, 2009). MARV was the first filovirus to be identified during an outbreak of Marburg haemorrhagic fever (MHF) among workers in Marburg, Frankfurt and Belgrade vaccine production facilities (Siegert *et al.*, 1967; Martini, 1969). The source of the infection was linked to African vervet (green) monkeys that were shipped from a facility in Lake Victoria, Uganda, Eastern Africa, but the actual source of infection of the monkeys could not be determined (Siegert *et al.*, 1967; Mahanty and Bray, 2004). This outbreak resulted in 31 cases with a 23% fatality rate and constitutes the largest human filovirus haemorrhagic fever (FHF) episode outside the filovirus endemic region of central Africa (see Table 15.1).

The next MARV outbreak occurred in Johannesburg, South Africa, where the index case was an Australian hitchhiker who contracted the disease, likely during his visit through Zimbabwe in February 1975 (Gear *et al.*, 1975). This was the first diagnosed MHF acquired in natural settings and included two secondary infections, a travel companion who developed the symptoms 2 days after the index case died and a nursing sister who took care of the index case (Gear *et al.*, 1975). The two secondary cases survived the illness. A series of serum samples from humans and animals, and even different Arthropod samples, were collected in an effort to investigate the source of infection to the index case, but no antibodies against marburgvirus were detected and no marburgvirus grew from Arthropod extracts inoculated in tissue cultures (Conrad *et al.*, 1978).

In 1980, a single natural acquired fatal case of MHF was reported in Kenya. The attending physician was infected with the virus but survived the severe disease (Smith *et al.*, 1982). Seven years later, another fatal case of MHF occurred in the same region of western Kenya. Further characterization of this case resulted in the isolation of the RAVV, the second member of the *Marburg marburgvirus*, diverging around 21%, at the nucleotide level, from the Lake Victoria type species (Johnson *et al.*, 1996; Towner *et al.*, 2006). In both Kenyan outbreaks, the source of infection was associated with visiting Kitum Cave, where Egyptian fruit bats (*Rousettus aegyptiacus*) were diagnosed positive for MARV nucleoprotein gene (Kuzmin *et al.*, 2010).

The sporadic, low fatality rate and small size of the MHF outbreaks gave the erroneous view that MARV did not have the epidemiological relevance of its counterpart EBOV, which by the year 2000 had caused around 19 outbreaks of Ebola haemorrhagic fever (EHF) (Cardenas, 2010). This notion was drastically changed during the first large community-based outbreak of MHF in the localities of Durba and Watsa, Democratic

Table 15.1. Marburgvirus natural outbreaks history. (Data adapted from Cardenas (2010) with permission.)

No.	Location	Year	Human cases (deaths)	Case fatality rate (%)
1	Germany (Marburg and Frankfurt), former Yugoslavia (Belgrade) (Siegert *et al*., 1967)	1967	31 (7)	23
2	South Africa (Johannesburg) (Gear *et al*., 1975)	1975	3 (1)	33
3	Kenya (Mount Elgon National Park) (Smith *et al*., 1982)	1980	2 (1)	50
4	Kenya (Mount Elgon National Park) (Johnson *et al*., 1996)	1987	1 (1)	100
5	DRC (Durba, gold mine village) (Bausch *et al*., 2006)	1998–2000	154 (128)	83
6	Angola (countrywide, but largely from Uige Province) (WHO, 2005)	2004–2005	374 (329)	88
7	Uganda (mine workers in Kakasi Forest Reserve, Kamwenge District) (WHO, 2007)	2007	4 (1)	25
8	Uganda (US tourist at Maramagambo Forest, Phyton Cave) (CDC, 2009)	2008	1 (0)	0
9	Uganda (Dutch tourist at Maramagambo Forest, Phyton Cave) (Timen *et al*., 2009)	2008	1 (1)	100
10[a]	Uganda (reported from four districts, namely Kabale, Kampala, Ibanda and Mbarara) (WHO, Global Alert and Response 23 November 2012)	2012	20 (9)	45

[a] At the time of writing this chapter, a new MHF outbreak had been declared on 19 October 2012, with the last confirmed case reported on 31 October 2012.

Republic of the Congo (DRC), where 154 cases were reported over 2 years, from 1998 to 2000, with an accumulated case fatality of 83% (Bausch *et al*., 2006). This outbreak was characterized by the introduction of multiple genetically distinct lineages of MARV, possibly linked to a close contact with the virus reservoir species during underground mining activity. Interestingly, it seems that the disease around the Durba mines was present since 1987, where it was known as the 'haemorrhagic syndrome of Durba'. A 1994 survivor from the syndrome had antibodies against MARV (Swanepoel *et al*., 2007). Investigation of different cave-dwelling organisms to identify the source of the Durba-Watsa outbreak identified three bat species positive for MARV nucleic acids (Swanepoel *et al*., 2007). Two insectivorous bats, *Miniopterus inflatus* and *Rhinolophus eloquens*, were positive for MARV VP35 gene, but antibodies against MARV was only detected in *R. eloquens*. The third species, *R. aegyptiacus*, were positive for VP35 and antibodies. Further phylogenetic analysis of the VP35 amplicons indicated the presence of virus variants, with some of them matching the sequences of the human MARV isolates, implying an epidemiological link between the MHF outbreak and the bat population roosting in the cave (Swanepoel *et al*., 2007).

But the scientific community was surprised again when a new outbreak of MHF reappeared with more than the double cases of the Durba-Watsa outbreak, and for the first time in western Africa, where EBOV is the prevalent cause of FHF. MARV infection hit the northern province of Angola, Uige, with an estimated 374 cases and a case fatality

of 88% (WHO, 2005). Contrary to the Durba-Watsa outbreak, the Angolan isolates did not show major nucleotide differences, which indicates that the outbreak likely started with the introduction of a single strain from the reservoir species followed by person-to-person transmission (Towner *et al.*, 2006). In June–July 2007, an outbreak of MHF was reported among mineworkers in the Ibanda district, western Uganda. The index case who initially took care of a co-worker with symptoms consistent with haemorrhagic fever contracted the illness and died a few days later. A third co-worker who had earlier developed an apparently mild form of MHF helped the index case had cared for their partner. All three cases were laboratory diagnosed with MARV. A fourth case that slept at the mine camp was diagnosed with MARV in September of the same year (WHO, 2007; Adjemian *et al.*, 2011). Investigation of the bats roosting in the mine as the possible source of MARV infection showed that 2.6% of the sampled population were positive for MARV as assessed by VP40 Q-RT-PCR and a subset of these were also positive for NP-VP35 by conventional RT-PCR (Towner *et al.*, 2009). As with the bat surveillance in the Durba mine, the frugivorous bat *R. aegyptiacus* was positive for MARV. In the last outbreak site, virus isolation was only possible in samples that were tested positive by both Q-RT-PCR and conventional RT-PCR. Phylogenetic analysis of full-length virus genome from *R. aegyptiacus* and two human isolates from the Ibanda district MHF outbreak showed that one human isolate and two of the bat isolates fell into the clade that grouped most of the prototype MARV. The second human isolate fell into the clade that grouped the RAVV lineage, together with three of the bat isolates (Towner *et al.*, 2009). This indicates that there were at least two independent virus lineages introduced in the Ibanda outbreak by the natural reservoir hosts. Phylogenetic clustering of the human and bat MARV sequences in the Durba and the Ibanda outbreaks indicate that bats, especially the Egyptian fruit bat, are the natural reservoir of MARV. Two MHF outbreaks that were imported individually to the USA and the Netherlands were linked to independent visits to the Phyton Cave, Uganda, where a large population of *R. aegyptiacus* live.

Although there is strong evidence that points to the Egyptian fruit bat, and possibly some insectivorous bats, as the natural reservoirs of MARV, it is still unclear how they transmit the virus to susceptible hosts because no viral RNA has been detected in oral swabs, urine or faeces, or in blood or placenta samples (Towner *et al.*, 2009). However, it is not surprising that the initial attempts to detect the mechanisms of infection were not conclusive because MHF outbreaks are rare events and only a small percentage (3–5%) of the bat population are shown to be positive by the most sensitive Q-RT-PCR diagnosis and a fraction of those positives are able to produce virus isolates. In addition, the complex ecology of the bat population, including age, reproductive status, breeding habits, migrations, colonies interactions and seasonal behaviour, can make it difficult to identify the right ecological, environmental and biological conditions for virus transmission. This contrasts with a previous report where EBOV was detected in faeces and lung tissue of experimentally inoculated insectivorous and fruit bats (Swanepoel *et al.*, 1996). However, further experiments demonstrating viral transmission with infectious nasal secretions or faeces have not been done. Also, to our knowledge, we cannot rule out the presence of an intermediary host between bats and humans and non-human primates.

15.2.2 Host range

The host range of filovirus includes humans, non-human primates, bats, duikers and swine (Leroy *et al.*, 2004; Barrette *et al.*, 2009). Except for LLOV, all filovirus cause a highly fatal FHF in non-human primates, which has been associated with the dramatic decrease of gorilla and chimpanzee natural populations in Central Africa (Leroy *et al.*, 2004). Reston ebolavirus, discovered in infected cynomolgus monkeys (*Macaca*

fascicularis) imported from the Philippines to a quarantine facility in Reston, Virginia, USA, in 1989, is the only member of the *Ebolavirus* that has been associated with severe disease in non-human primates and swine, but not in humans (Jahrling *et al.*, 1990; Hayes *et al.*, 1992; WHO, 1992; Miranda *et al.*, 1999; Rollin *et al.*, 1999). Interestingly, several animal handlers at the export facility in the Philippines and in the different outbreak locations showed seroconversion without any apparent Ebola-like illness (Centers for Disease Control and Prevention, 1990; Miranda *et al.*, 1991, 1999). Marburgvirus has only been reported to cause severe disease in human and non-human primates (see Table 15.1). Except for the first MHF outbreak in Europe, all infections can be linked to a bat source (Table 15.2) (Towner *et al.*, 2009). So far, all bats that have been diagnosed to contain either MARV nucleic acids or MARV IgG antibodies have not shown signs of illness consistent with FHF. This agrees with the lack of apparent FHF in experimentally EBOV-inoculated insectivorous (*Tadaria pumila*) and fruit (*Epomophorus wahlbergi*) bats (Swanepoel *et al.*, 1996).

In laboratory conditions, besides the African green monkey, other non-human primates were shown to be susceptible to MARV infection and to develop MHF disease. The rhesus macaque (*Macaca mulatta*) (Simpson *et al.*, 1968; Simpson, 1969; Zlotnik, 1969; Johnson *et al.*, 1996; Daddario-DiCaprio *et al.*, 2006; Geisbert *et al.*, 2007), cynomolgus macaque (*Macaca fascicularis*) (Jones *et al.*, 2005; Hensley *et al.*, 2011) and squirrel monkey (*Saimiri sciureus*) (Simpson, 1969; Zlotnik, 1969) have all been used in experimental MARV infections and developed MHF with almost homogeneously fatal outcomes. Guinea pigs were also susceptible to MARV infection, although an initial cycle of febrile illness was observed with a human-derived virus, followed by a slow recovery of health. However, subsequent inoculations with guinea pig-derived virus resulted in a shorter incubation period and a severe disease with fatal outcomes (Simpson *et al.*, 1968). After several passages of the virus in guinea pigs, mortality can reach >90%. This increase in mortality rate was associated with the adaptation of the virus to the new host by acquiring mutations in the coding and non-coding regions of the

Table 15.2. Marburgvirus infections in animals, acquired under non-experimental conditions.

Host	Place	Test	Year
Non-human primates	Marburg, Frankfurt, Belgrade	Electron microscopy	1967 (Siegert *et al.*, 1967)
African green (velvet) monkey (*Cercopithecus thiops*)			
Bats	Goroumbwa Mine, Durba, Democratic Republic of the Congo	RT-PCR of L; nested RT-PCR of VP35; Marburg IgG	1999 (Swanepoel *et al.*, 2007)
Miniopterus inflatus			
Rhinolophus eloquens			
Rousettus aegyptiacus			
Rousettus aegyptiacus	Near caves in Western Gabon	qRT-PCR of VP40; nested RT-PCR of VP35 & NP; Marburg IgG	2005, 2006 (Towner *et al.*, 2007)
Rousettus aegyptiacus	Kitaka cave, near Ibanda village, western Uganda	qRT-PCR of VP40; nested RT-PCR of VP35 & NP; Marburg IgG	2007, 2008 (Towner *et al.*, 2009)
Hipposideros spp.			
Rousettus aegyptiacus	Kitum cave, western Kenya	Nested RT-PCR of NP	2007 (Kuzmin *et al.*, 2010)
Rousettus aegyptiacus	Zadié Cave y Batouala Cave, near Belinga village, Gabon	qRT-PCR of VP40; nested RT-PCR of VP35	2009, 2010 (Maganga *et al.*, 2011)

MARV genome (Lofts *et al.*, 2007). Similar increase in virus pathogenicity was observed in mouse-adapted MARV, where nucleotide changes were detected in the UTR regions of NP, VP35, VP40, GP, VP30 and in all ORFs except for VP24 (Lofts *et al.*, 2011). Although these mutations allowed the onset of MHF-like illness in these animals, different viral strains required specific mutations that resulted in different degrees of pathogenicity.

15.2.3 Pathology and pathogenesis

All filoviruses produce a similar illness in human and non-human primates once the symptoms appear, but differ in the severity and case fatality rate, depending on the virus species and virus strain involved (Mahanty and Bray, 2004; Ascenzi *et al.*, 2008). Although marburgvirus was the first filovirus to be discovered, FHF caused by ebolavirus has been extensively studied in natural and laboratory settings. Notwithstanding the paucity of information, the fact that the first FHF outbreak occurred in a well-resourced western clinical environment permitted a detailed account of the MHF epidemic's clinical evolution (Martini, 1969). From the clinical records of at least 23 patients, it was determined that the time between the initial contact with the infectious material until the first symptoms appeared was 5–7 days. The illness period ranged from 7 to 26 days in surviving cases and from 7 to 16 days in fatal cases. Initial symptoms were headaches, malaise and myalgia; vomiting and diarrhoea peaked around the 4th and 6th day of symptom onset, respectively; lymphoadenopathy and drowsiness was observed in most patients around the 6th day; diarrhoea continued while enanthem and a rash appeared in most patients between the 7th and 8th day. Pyrexia was observed on the first day, then briefly diminished to increase again from day 3 to day 6 and then decreased steadily until day 18. The most reliable and characteristic sign of the illness was a rash on different parts of the body that began with pinhead dark red papules around the hair follicles that became maculopapular, sharply delineated and later coalesced into a diffuse rash (Martini, 1969). All patients developed severe thrombocytopenia leading to haemorrhage from the gums, nose, puncture lesions and into the gastrointestinal tract with haematemesis and melaena. However, measurement of different coagulation factors did not show out of the range values, contrary to coagulation abnormalities reported for EHF (Geisbert *et al.*, 2003). All patients showed an increase of serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) with a peak increase around the 7th and 8th day. Fatal cases were associated with very high levels of SGOT, implying damage to the liver, muscle tissue or other organs. Tachycardia and high temperature were present in all fatal cases. A similar clinical manifestation was observed in the Durba-Watsa MHF outbreak, except that higher haemorrhagic symptoms and fatal outcomes were present than in the Marburg outbreak. Fatal cases in the DRC outbreak were associated with a significant increased in diarrhoea, bloody diarrhoea, conjuntivitis, hiccups and bleeding from injection sites, but a decrease in chest pain (Bausch *et al.*, 2006; Colebunders *et al.*, 2007). It is likely that different clinical supportive care between the European and the DRC outbreaks influenced the disease evolution and outcomes. Among other factors, the virus strain pathogenicity could also have influenced the case fatality rate. Indeed, experimental infection of adult rhesus macaques (*Macaca mulatta*) with the Angolan strain of MARV showed a rapid MHF disease progression and higher effects on their livers as compared with Marburg virus (i.e. Musoke type strain) (Geisbert *et al.*, 2007).

In general there is limited clinical information available for MHF disease progression and its pathophysiological mechanisms (Mehedi *et al.*, 2011). At the molecular level, most of our detailed knowledge of virus–host interactions comes from laboratory non-human primate, guinea pig, mouse and *in vitro* models of the disease. Experimental evidence in cynomolgus

macaques showed that, as was demonstrated with EBOV infections, antigen presenting cells are the initial targets of MARV infection (Hensley *et al.*, 2011). Inhibition of proper cell maturation and downregulation of key cellular surface immune receptors due to viral replication in these cells prevented an adequate innate immune response and subsequent adaptive cell-mediated and humoral responses. Dendritic cells (DCs), professional antigen presenting cells with a key role in activating and modulating the initial innate immune response and naïve T cell stimulation (Geijtenbeek *et al.*, 2000; Liu, 2001) can support MARV and EBOV replication without inducing cytokines secretion (Bosio *et al.*, 2003). *In vitro* infections of human monocyte-derived DCs with MARV or EBOV resulted in the impairment of cytokine secretion, including interferon (IFN)-α, when exposed to a source of double-stranded (ds) RNA. This effect was specific to viral activation pathways because lipopolyssacharide treatment stimulated infected DCs to secrete TNF-α (Bosio *et al.*, 2003). On the other hand, infections of monocytes or macrophages with MARV or EBOV resulted in the secretion of proinflammatory cytokines and chemokines like IL-1β, TNF-α, IL-6, IL8, gro-α and RANTES (Stroher *et al.*, 2001; Bosio *et al.*, 2003). It has been proposed that the high degree of immune activation, assessed by the increase of serum cytokines and chemokines, contributes to MHF and EHF pathogenesis by inducing vascular dysfunction, hypotension and multiple organ failure (Mahanty and Bray, 2004). However, the timing of proinflammatory mediator appearance after infection and their control by antagonist cytokines have been shown to correlate with disease outcome (Baize *et al.*, 1999, 2002; Leroy *et al.*, 2000, 2001). In MARV-infected cynomolgus monkeys, increased levels of IFN-α, IL-6, MIP-1α, MIP-1β, MCP-1, eotaxin, IFN-γ, IL-1R, IL-2R, IL-8, IL-12 p40/p70, IL-13 and TNF-α were observed at later stages (days 6–8) of the disease (Hensley *et al.*, 2011). Experimental evidence suggests that most of these secreted cytokines originate from monocytes and macrophages and that MARV or EBOV VP35 is responsible for host immune depression by inhibiting DC activation and IFN-α secretion (Gupta *et al.*, 2001; Bosio *et al.*, 2003; Hensley *et al.*, 2011).

Type I interferons (IFNα/β) are cytokines at the centre of the innate antiviral cellular response and key modulators of the adaptive immunity (Weber *et al.*, 2004; Haller and Weber, 2007; Swiecki and Colonna, 2011; MacMicking, 2012). Viral activation of the signaling cascade leading to IFN-I secretion will promote an antiviral state in the infected and neighbouring cells. Thus, it is not surprising that viruses have evolved different strategies to subvert the antiviral response mediated by IFN-I to secure their replication and dissemination (Haller and Weber, 2007). The ability of MARV to infect and cause severe illness in a mouse model of FHF that was defective in IFN-I receptor (–/–), but not in a normal BALBc, was the initial evidence that implicated IFN-I in the host immune defence against MARV infection (Bray, 2001). A global gene expression study with human hepatoblastoma (Huh7) cells infected with MARV showed that the virus was able to suppress key antiviral host responses, which included IFN-stimulated genes (ISGs) via the Jak-STAT pathway (Kash *et al.*, 2006). Indeed, Huh7 cells previously infected with MARV and treated with IFN-I showed a strong reduction in STAT1 and 2 phosphorylation as compared to mock infected Huh7 control cells. Further investigation on the mechanisms of STAT1 and 2 phosphorylation inhibition identified the MARV major matrix protein VP40 as the IFN-antagonist protein responsible for inhibiting IFN-I signaling by targeting the Janus kinases, Jak1 and Tyk2 (Valmas *et al.*, 2010). Because the Jaks are also involved in IFN-II and IL6 cellular signaling through STAT1 and STAT3, MARV VP40 also blocked these pathways by inhibiting STAT1 and -3 phosphorylation. Surprisingly, the IFN-I and -II signal antagonist protein in ebolaviruses is not the major matrix protein, but the minor matrix protein VP24 (Reid *et al.*, 2006, 2007; Mateo *et al.*, 2010). Although VP24 does not impair STAT1 phosphorylation by the Janus kinases, it can effectively block ISGs transcription by impeding phosphorylated STAT1 to interact

with karyopherins α1, α5 and α6 and the nuclear translocation of the complex. It is intriguing that, regardless of the ability of MARV and EBOV to control the host gene expression to antagonize the cellular innate antiviral responses, ebolavirus IFN-I antagonist protein, VP35 (Basler *et al.*, 2000; Leung *et al.*, 2010b), seems to lack its counterpart in marburgviruses. But experiments with human monocytes-derived DCs, infected with Venezuelan equine encephalitis replicon particles (VRPs) encoding MARV VP35 or EBOV VP35, did not induce IFN-I production as compared to control VRP encoding green fluorescent protein (Bosio *et al.*, 2003). This suggests an IFN-I antagonist function for MARV and EBOV VP35 proteins. On the other hand, MARV VP35 showed a weak inhibition of IFN-β reporter gene activation by Sendai virus infection of HEK-293T, as compared to Ebola virus and Reston virus VP35 proteins (Bale *et al.*, 2012). Crystal structure analysis of Ebola virus, Reston virus and Marburg virus VP35 C-terminal domain gave structural evidence of previous functional reports of its double-stranded RNA (dsRNA)-binding capacity (Cardenas *et al.*, 2006; Leung *et al.*, 2010a, 2010b; Bale *et al.*, 2012). Functional characterization of VP35 mutants, at key basic residues required for dsRNA-binding, demonstrated residual IFN-antagonist activity implying a dsRNA-binding independent IFN-antagonist function of VP35. It is likely that as more molecular virus–host interaction components are discovered, the mechanisms of MARV, and other filoviruses, antiviral evasion strategies will be unravelled.

15.3 Marburgvirus Diagnostics

All diagnostics of MHF include a combination of three main techniques or variations of them: clinical case definition, enzyme-linked immunosorbent assay (ELISA) and reverse transcriptase polymerase chain reaction (RT-PCR). Even with new technologies that are more sensitive, with multiplex capabilities and with high throughput applications, a good clinical assessment is always critical to detect early signs of FHF-like illness (Hartman *et al.*, 2010). In 2005, during the largest MHF outbreak in Angola, western central Africa, the WHO outbreak response team deployed a mobile laboratory unit (MLU) that successfully implemented a Q-RT-PCR diagnostic of MARV, from swab and EDTA blood samples, in less than 4 hours per sample (Grolla *et al.*, 2011). The diagnostic target genes were L and NP, with a one-step assay set up to reduce contamination, especially in field conditions. Confirmatory tests like antigen capture ELISA and Q-RT-PCR of the VP40 gene were performed in the regional reference laboratory. Virus isolation and genome sequencing have an important value for epidemiological investigation but are not practical for field conditions. It is important to note that the genetic diagnostic should target genes and regions of high conservacy to accommodate virus genetic variations, but be specific enough to avoid crossreaction with related virus families. The use of SyBR Green-based RT-PCR is suitable for its sensitivity and specificity in field conditions at a low cost (Paudel *et al.*, 2011).

15.4 Concluding Remarks

It has been 45 years since the first outbreak of MHF occurred in European vaccine facilities. The last MHF outbreak was reported in Uganda, Africa in 2012, with a total of 10 recorded outbreaks. However, the paucity of clinical and immunological information prevent us from better understanding the molecular basis of MARV pathogenicity and disease outcome. It is likely that the recent increasing epidemiological importance that EBOV has acquired, because of the frequency, size and high fatality rates of EHF outbreaks, has resulted in the overshadowing of the importance of MARV as an emerging pathogen. The recent report on the first IFN-I, IFN-II and IL6 signaling pathway antagonist MARV protein will pave the way to exciting new investigations to elucidate the different host signaling components that interact with or are affected by the viral

protein. This is the case for Ebola virus VP35 and VP24 proteins. Even with the need for more research on filovirus–host interactions, parallel investigations on vaccine platforms to protect susceptible mammalian hosts to FHF have resulted in promising vaccine candidates that offer good immune protection to non-human primates (Jones *et al.*, 2005; Sullivan *et al.*, 2006; Warfield *et al.*, 2007). The recent discovery of Reston virus (RSTV) associated with a porcine reproductive and respiratory disease syndrome (PRRS) in the Philippines is of particular concern because of its epidemiological implications and human livestock risks (Barrette *et al.*, 2009). Although FHF outbreaks have mainly occurred in central Africa, where it represents an important public and wildlife health concern, the importation of human cases to the USA and Europe, the new filovirus detected in dead bats from Spain and the association of RSTV with epidemics of PRRS in Asia, clearly underscore the global health impact that filoviruses could have as emerging human and animal pathogens. Thus further research on the molecular mechanisms of host antiviral evasion and vaccine development should be guaranteed in order to support current public health surveillance and outbreak control efforts.

References

Adjemian, J., Farnon, E.C., Tschioko, F., Wamala, J.F., Byaruhanga, E., Bwire, G.S., Kansiime, E., Kagirita, A., Ahimbisibwe, S., Katunguka, F., Jeffs, B., Lutwama, J.J., Downing, R., Tappero, J.W., Formenty, P., Amman, B., Manning, C., Towner, J., Nichol, S.T. and Rollin, P.E. (2011) Outbreak of Marburg hemorrhagic fever among miners in Kamwenge and Ibanda Districts, Uganda, 2007. *Journal of Infectious Diseases* 204, S796–799.

Ascenzi, P., Bocedi, A., Heptonstall, J., Capobianchi, M.R., Di Caro, A., Mastrangelo, E., Bolognesi, M. and Ippolito, G. (2008) Ebolavirus and Marburgvirus: insight the Filoviridae family. *Molecular Aspects of Medicine* 29, 151–185.

Baize, S., Leroy, E.M., Georges-Courbot, M.C., Capron, M., Lansoud-Soukate, J., Debre, P., Fisher-Hoch, S.P., McCormick, J.B. and Georges, A.J. (1999) Defective humoral responses and extensive intravascular apoptosis are associated with fatal outcome in Ebola virus-infected patients. *Nature Medicine* 5, 423–426.

Baize, S., Leroy, E.M., Georges, A.J., Georges-Courbot, M.C., Capron, M., Bedjabaga, I., Lansoud-Soukate, J. and Mavoungou, E. (2002) Inflammatory responses in Ebola virus-infected patients. *Clinical and Experimental Immunology* 128, 163–168.

Bale, S., Julien, J.P., Bornholdt, Z.A., Kimberlin, C.R., Halfmann, P., Zandonatti, M.A., Kunert, J., Kroon, G.J., Kawaoka, Y., Macrae, I.J., Wilson, I.A. and Saphire, E.O. (2012) Marburg virus VP35 can both fully coat the backbone and cap the ends of dsRNA for interferon antagonism. *PLoS Pathogen* 8, e1002916.

Barrette, R.W., Metwally, S.A., Rowland, J.M., Xu, L., Zaki, S.R., Nichol, S.T., Rollin, P.E., Towner, J.S., Shieh, W.J., Batten, B., Sealy, T.K., Carrillo, C., Moran, K.E., Bracht, A.J., Mayr, G.A., Sirios-Cruz, M., Catbagan, D.P., Lautner, E.A., Ksiazek, T.G., White, W.R. and McIntosh, M.T. (2009) Discovery of swine as a host for the Reston ebolavirus. *Science* 325, 204–206.

Basler, C.F., Wang, X., Mühlberger, E., Volchkov, V., Paragas, J., Klenk, H.D., Garcia-Sastre, A. and Palese, P. (2000) The Ebola virus VP35 protein functions as a type I IFN antagonist. *Proceedings of the National Academy of Sciences of the USA* 97, 12,289–12,294.

Bausch, D.G., Nichol, S.T., Muyembe-Tamfum, J.J., Borchert, M., Rollin, P.E., Sleurs, H., Campbell, P., Tshioko, F.K., Roth, C., Colebunders, R., Pirard, P., Mardel, S., Olinda, L.A., Zeller, H., Tshomba, A., Kulidri, A., Libande, M.L., Mulangu, S., Formenty, P., Grein, T., Leirs, H., Braack, L., Ksiazek, T., Zaki, S., Bowen, M.D., Smit, S.B., Leman, P.A., Burt, F.J., Kemp, A. and Swanepoel, R. (2006) Marburg hemorrhagic fever associated with multiple genetic lineages of virus. *New England Journal of Medicine* 355, 909–919.

Bosio, C.M., Aman, M.J., Grogan, C., Hogan, R., Ruthel, G., Negley, D., Mohamadzadeh, M., Bavari, S. and Schmaljohn, A. (2003) Ebola and Marburg viruses replicate in monocyte-derived dendritic cells without inducing the production of cytokines and full maturation. *Journal of Infectious Diseases* 188, 1630–1638.

Bray, M. (2001) The role of the Type I interferon response in the resistance of mice to filovirus infection. *Journal of General Virology* 82, 1365–1373.

Cardenas, W.B. (2010) Evasion of the interferon-mediated antiviral response by filovirus. *Viruses* 2, 262–282.

Cardenas, W.B., Loo, Y.M., Gale, M. Jr, Hartman, A.L., Kimberlin, C.R., Martinez-Sobrido, L., Saphire, E.O. and Basler, C.F. (2006) Ebola virus VP35 protein binds double-stranded RNA and inhibits alpha/beta interferon production induced by RIG-I signaling. *Journal of Virology* 80, 5168–5178.

CDC (2009) Imported case of Marburg hemorrhagic fever – Colorado, 2008. *Morbidity and Mortality Weekly Report* 58, 1377–1381.

Centers for Disease Control and Prevention (1990) Update: evidence of filovirus infection in an animal caretaker in a research/service facility. *Morbidity and Mortality Weekly Report* 39, 296–297.

Colebunders, R., Tshomba, A., Van Kerkhove, M.D., Bausch, D.G., Campbell, P., Libande, M., Pirard, P., Tshioko, F., Mardel, S., Mulangu, S., Sleurs, H., Rollin, P.E., Muyembe-Tamfum, J.J., Jeffs, B. and Borchert, M. (2007) Marburg hemorrhagic fever in Durba and Watsa, Democratic Republic of the Congo: clinical documentation, features of illness, and treatment. *Journal of Infectious Diseases* 196, S148–153.

Conrad, J.L., Isaacson, M., Smith, E.B., Wulff, H., Crees, M., Geldenhuys, P. and Johnston, J. (1978) Epidemiologic investigation of Marburg virus disease, Southern Africa, 1975. *American Journal of Tropical Medicine and Hygiene* 27, 1210–1215.

Daddario-DiCaprio, K.M., Geisbert, T.W., Stroher, U., Geisbert, J.B., Grolla, A., Fritz, E.A., Fernando, L., Kagan, E., Jahrling, P.B., Hensley, L.E., Jones, S.M. and Feldmann, H. (2006) Postexposure protection against Marburg haemorrhagic fever with recombinant vesicular stomatitis virus vectors in non-human primates: an efficacy assessment. *Lancet* 367, 1399–1404.

Feldmann, H., Mühlberger, E., Randolf, A., Will, C., Kiley, M.P., Sanchez, A. and Klenk, H.D. (1992) Marburg virus, a filovirus: messenger RNAs, gene order, and regulatory elements of the replication cycle. *Virus Research* 24, 1–19.

Gear, J.S., Cassel, G.A., Gear, A.J., Trappler, B., Clausen, L., Meyers, A.M., Kew, M.C., Bothwell, T.H., Sher, R., Miller, G.B., Schneider, J., Koornhof, H.J., Gomperts, E.D., Isaacson, M. and Gear, J.H. (1975) Outbreak of Marburg virus disease in Johannesburg. *British Medical Journal* 4, 489–493.

Geijtenbeek, T.B., Torensma, R., van Vliet, S.J., van Duijnhoven, G.C., Adema, G.J., van Kooyk, Y. and Figdor, C.G. (2000) Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell* 100, 575–585.

Geisbert, T.W., Young, H.A., Jahrling, P.B., Davis, K.J., Kagan, E. and Hensley, L.E. (2003) Mechanisms underlying coagulation abnormalities in Ebola hemorrhagic fever: overexpression of tissue factor in primate monocytes/macrophages is a key event. *Journal of Infectious Diseases* 188, 1618–1629.

Geisbert, T.W., Daddario-DiCaprio, K.M., Geisbert, J.B., Young, H.A., Formenty, P., Fritz, E.A., Larsen, T. and Hensley, L.E. (2007) Marburg virus Angola infection of rhesus macaques: pathogenesis and treatment with recombinant nematode anticoagulant protein c2. *Journal of Infectious Diseases* 196, S372–381.

Grolla, A., Jones, S.M., Fernando, L., Strong, J.E., Stroher, U., Moller, P., Paweska, J.T., Burt, F., Pablo Palma, P., Sprecher, A., Formenty, P., Roth, C. and Feldmann, H. (2011) The use of a mobile laboratory unit in support of patient management and epidemiological surveillance during the 2005 Marburg Outbreak in Angola. *PLoS Neglected Tropical Diseases* 5, e1183.

Gupta, M., Mahanty, S., Ahmed, R. and Rollin, P.E. (2001) Monocyte-derived human macrophages and peripheral blood mononuclear cells infected with ebola virus secrete MIP-1alpha and TNF-alpha and inhibit poly-IC-induced IFN-alpha in vitro. *Virology* 284, 20–25.

Haller, O. and Weber, F. (2007) Pathogenic viruses: smart manipulators of the interferon system. *Current Topics in Microbiology and Immunology* 316, 315–334.

Hartman, A.L., Towner, J.S. and Nichol, S.T. (2010) Ebola and Marburg hemorrhagic fever. *Clinics in Laboratory Medicine* 30, 161–177.

Hayes, C.G., Burans, J.P., Ksiazek, T.G., Del Rosario, R.A., Miranda, M.E., Manaloto, C.R., Barrientos, A.B., Robles, C.G., Dayrit, M.M. and Peters, C.J. (1992) Outbreak of fatal illness among captive macaques in the Philippines caused by an Ebola-related filovirus. *American Journal of Tropical Medicine and Hygiene* 46, 664–671.

Hensley, L.E., Alves, D.A., Geisbert, J.B., Fritz, E.A., Reed, C., Larsen, T. and Geisbert, T.W. (2011) Pathogenesis of Marburg hemorrhagic fever in cynomolgus macaques. *Journal of Infectious Diseases* 204, S1021–1031.

Ikegami, T., Calaor, A.B., Miranda, M.E., Niikura, M., Saijo, M., Kurane, I., Yoshikawa, Y. and Morikawa, S. (2001) Genome structure of Ebola virus subtype Reston: differences among Ebola subtypes. Brief report. *Archives of Virology* 146, 2021–2027.

Jahrling, P.B., Geisbert, T.W., Dalgard, D.W., Johnson, E.D., Ksiazek, T.G., Hall, W.C. and Peters, C.J. (1990) Preliminary report: isolation of Ebola virus from monkeys imported to USA. *Lancet* 335, 502–505.

Johnson, E.D., Johnson, B.K., Silverstein, D., Tukei, P., Geisbert, T.W., Sanchez, A.N. and Jahrling, P.B. (1996) Characterization of a new Marburg virus isolated from a 1987 fatal case in Kenya. *Archives of Virology Supplementum* 11, 101–114.

Jones, S.M., Feldmann, H., Stroher, U., Geisbert, J.B., Fernando, L., Grolla, A., Klenk, H.D., Sullivan, N.J., Volchkov, V.E., Fritz, E.A., Daddario, K.M., Hensley, L.E., Jahrling, P.B. and Geisbert, T.W. (2005) Live attenuated recombinant vaccine protects nonhuman primates against Ebola and Marburg viruses. *Nature Medicine* 11, 786–790.

Kash, J.C., Mühlberger, E., Carter, V., Grosch, M., Perwitasari, O., Proll, S.C., Thomas, M.J., Weber, F., Klenk, H. and Katze, M.G. (2006) Global suppression of the host antiviral response by Ebola- and Marburgviruses: increased antagonism of the type I interferon response is associated with enhanced virulence. *Journal of Virology* 80, 3009–3020.

Kuhn, J.H., Becker, S., Ebihara, H., Geisbert, T.W., Johnson, K.M., Kawaoka, Y., Lipkin, W.I., Negredo, A.I., Netesov, S.V., Nichol, S.T., Palacios, G., Peters, C.J., Tenorio, A., Volchkov, V.E. and Jahrling, P.B. (2010) Proposal for a revised taxonomy of the family Filoviridae: classification, names of taxa and viruses, and virus abbreviations. *Archives of Virology* 155, 2083–2103.

Kuzmin, I.V., Niezgoda, M., Franka, R., Agwanda, B., Markotter, W., Breiman, R.F., Shieh, W.J., Zaki, S.R. and Rupprecht, C.E. (2010) Marburg virus in fruit bat, Kenya. *Emerging Infectious Diseases* 16, 352–354.

Lamb, R.A. (2007) Mononegavirales. In: Knipe, D.M. and Howley, P.M. (eds) *Fields Virology*. Lippincott Williams & Wilkins, Philadelphia, Pennsylvania, pp. 1357–1361.

Le Guenno, B., Formenty, P., Wyers, M., Gounon, P., Walker, F. and Boesch, C. (1995) Isolation and partial characterisation of a new strain of Ebola virus. *Lancet* 345, 1271–1274.

Leroy, E.M., Baize, S., Volchkov, V.E., Fisher-Hoch, S.P., Georges-Courbot, M.C., Lansoud-Soukate, J., Capron, M., Debre, P., McCormick, J.B. and Georges, A.J. (2000) Human asymptomatic Ebola infection and strong inflammatory response. *Lancet* 355, 2210–2215.

Leroy, E.M., Baize, S., Debre, P., Lansoud-Soukate, J. and Mavoungou, E. (2001) Early immune responses accompanying human asymptomatic Ebola infections. *Clinical and Experimental Immunology* 124, 453–460.

Leroy, E.M., Rouquet, P., Formenty, P., Souquiere, S., Kilbourne, A., Froment, J.M., Bermejo, M., Smit, S., Karesh, W., Swanepoel, R., Zaki, S.R. and Rollin, P.E. (2004) Multiple Ebola virus transmission events and rapid decline of central African wildlife. *Science* 303, 387–390.

Leung, D.W., Prins, K.C., Borek, D.M., Farahbakhsh, M., Tufariello, J.M., Ramanan, P., Nix, J.C., Helgeson, L.A., Otwinowski, Z., Honzatko, R.B., Basler, C.F. and Amarasinghe, G.K. (2010a) Structural basis for dsRNA recognition and interferon antagonism by Ebola VP35. *Nature Structural & Molecular Biology* 17, 165–172.

Leung, D.W., Shabman, R.S., Farahbakhsh, M., Prins, K.C., Borek, D.M., Wang, T., Mühlberger, E., Basler, C.F. and Amarasinghe, G.K. (2010b) Structural and functional characterization of Reston Ebola virus VP35 interferon inhibitory domain. *Journal of Molecular Biology* 399, 347–357.

Liu, Y.J. (2001) Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. *Cell* 106, 259–262.

Lofts, L.L., Ibrahim, M.S., Negley, D.L., Hevey, M.C. and Schmaljohn, A.L. (2007) Genomic differences between guinea pig lethal and nonlethal Marburg virus variants. *Journal of Infectious Diseases* 196, S305–312.

Lofts, L.L., Wells, J.B., Bavari, S. and Warfield, K.L. (2011) Key genomic changes necessary for an in vivo lethal mouse marburgvirus variant selection process. *Journal of Virology* 85, 3905–3917.

MacMicking, J.D. (2012) Interferon-inducible effector mechanisms in cell-autonomous immunity. *Nature Reviews Immunology* 12, 367–382.

Maganga, G.D., Bourgarel, M., Ella, G.E., Drexler, J.F., Gonzalez, J.P., Drosten, C. and Leroy, E.M. (2011) Is Marburg virus enzootic in Gabon? *Journal of Infectious Diseases* 204, S800–803.

Mahanty, S. and Bray, M. (2004) Pathogenesis of filoviral haemorrhagic fevers. *Lancet Infectious Diseases* 4, 487–498.

Martini, G.A. (1969) Marburg agent disease: in man. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 63, 295–302.

Mateo, M., Reid, S.P., Leung, L.W., Basler, C.F. and Volchkov, V.E. (2010) Ebolavirus VP24 binding to karyopherins is required for inhibition of interferon signaling. *Journal of Virology* 84, 1169–1175.

Mehedi, M., Groseth, A., Feldmann, H. and Ebihara, H. (2011) Clinical aspects of Marburg hemorrhagic fever. *Future Virology* 6, 1091–1106.

Miranda, M.E., White, M.E., Dayrit, M.M., Hayes, C.G., Ksiazek, T.G. and Burans, J.P. (1991) Seroepidemiological study of filovirus related to Ebola in the Philippines. *Lancet* 337, 425–426.

Miranda, M.E., Ksiazek, T.G., Retuya, T.J., Khan, A.S., Sanchez, A., Fulhorst, C.F., Rollin, P.E., Calaor, A.B., Manalo, D.L., Roces, M.C., Dayrit, M.M. and Peters, C.J. (1999) Epidemiology of Ebola (subtype Reston) virus in the Philippines, 1996. *Journal of Infectious Diseases* 179, S115–119.

Mühlberger, E., Lötfering, B., Klenk, H.D. and Becker, S. (1998) Three of the four nucleocapsid proteins of Marburg virus, NP, VP35, and L, are sufficient to mediate replication and transcription of Marburg virus-specific monocistronic minigenomes. *Journal of Virology* 72, 8756–8764.

Mühlberger, E., Weik, M., Volchkov, V.E., Klenk, H.D. and Becker, S. (1999) Comparison of the transcription and replication strategies of Marburg virus and Ebola virus by using artificial replication systems. *Journal of Virology* 73, 2333–2342.

Negredo, A., Palacios, G., Vazquez-Moron, S., Gonzalez, F., Dopazo, H., Molero, F., Juste, J., Quetglas, J., Savji, N., de la Cruz Martinez, M., Herrera, J.E., Pizarro, M., Hutchison, S.K., Echevarria, J.E., Lipkin, W.I. and Tenorio, A. (2011) Discovery of an ebolavirus-like filovirus in Europe. *PLoS Pathogen* 7, e1002304.

Paudel, D., Jarman, R., Limkittikul, K., Klungthong, C., Chamnanchanunt, S., Nisalak, A., Gibbons, R. and Chokejindachai, W. (2011) Comparison of real-time SYBR green dengue assay with real-time taqman RT-PCR dengue assay and the conventional nested PCR for diagnosis of primary and secondary dengue infection. *North American Journal of Medical Sciences* 3, 478–485.

Pringle, C.R. (1997) The order Mononegavirales – current status. *Archives of Virology* 142, 2321–2326.

Reid, S.P., Leung, L.W., Hartman, A.L., Martinez, O., Shaw, M.L., Carbonnelle, C., Volchkov, V.E., Nichol, S.T. and Basler, C.F. (2006) Ebola virus VP24 binds karyopherin alpha1 and blocks STAT1 nuclear accumulation. *Journal of Virology* 80, 5156–5167.

Reid, S.P., Valmas, C., Martinez, O., Sanchez, F.M. and Basler, C.F. (2007) Ebola virus VP24 proteins inhibit the interaction of NPI-1 subfamily karyopherin alpha proteins with activated STAT1. *Journal of Virology* 81, 13,469–13,477.

Rollin, P.E., Williams, R.J., Bressler, D.S., Pearson, S., Cottingham, M., Pucak, G., Sanchez, A., Trappier, S.G., Peters, R.L., Greer, P.W., Zaki, S., Demarcus, T., Hendricks, K., Kelley, M., Simpson, D., Geisbert, T.W., Jahrling, P.B., Peters, C.J. and Ksiazek, T.G. (1999) Ebola (subtype Reston) virus among quarantined non-human primates recently imported from the Philippines to the United States. *Journal of Infectious Diseases* 179, S108–114.

Sanchez, A., Kiley, M.P., Holloway, B.P. and Auperin, D.D. (1993) Sequence analysis of the Ebola virus genome: organization, genetic elements, and comparison with the genome of Marburg virus. *Virus Research* 29, 215–240.

Sanchez, A., Trappier, S.G., Mahy, B.W., Peters, C.J. and Nichol, S.T. (1996) The virion glycoproteins of Ebola viruses are encoded in two reading frames and are expressed through transcriptional editing. *Proceedings of the National Academy of Sciences of the USA* 93, 3602–3607.

Sanchez, A., Geisbert, T.W. and Feldmann, H. (2007) Filoviridae: Marburg and Ebola viruses. In: Knipe, D.M. and Howley, P.M. (eds) *Fields Virology*. Lippincott Williams & Wilkins, Philadelphia, Pennsylvania, pp. 1409–1448.

Siegert, R., Shu, H.L., Slenczka, W., Peters, D. and Muller, G. (1967) On the etiology of an unknown human infection originating from monkeys. *Deutsche Medizinische Wochenschrift* 92, 2341–2343.

Simpson, D.I. (1969) Marburg agent disease: in monkeys. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 63, 303–309.

Simpson, D.I., Zlotnik, I. and Rutter, D. A. (1968) Vervet monkey disease. Experiment infection of guinea pigs and monkeys with the causative agent. *British Journal of Experimental Pathology* 49, 458–464.

Smith, C.E., Simpson, D.I., Bowen, E.T. and Zlotnik, I. (1967) Fatal human disease from vervet monkeys. *Lancet* 290, 1119–1121.

Smith, D.H., Johnson, B.K., Isaacson, M., Swanapoel, R., Johnson, K.M., Killey, M., Bagshawe, A., Siongok, T. and Keruga, W.K. (1982) Marburg-virus disease in Kenya. *Lancet* 319, 816–820.

Stroher, U., West, E., Bugany, H., Klenk, H.D., Schnittler, H.J. and Feldmann, H. (2001) Infection and activation of monocytes by Marburg and Ebola viruses. *Journal of Virology* 75, 11,025–11,033.

Sullivan, N.J., Geisbert, T.W., Geisbert, J.B., Shedlock, D.J., Xu, L., Lamoreaux, L., Custers, J.H., Popernack, P.M., Yang, Z.Y., Pau, M.G., Roederer, M., Koup, R.A., Goudsmit, J., Jahrling, P.B. and Nabel, G.J. (2006) Immune protection of nonhuman primates against Ebola virus with single low-dose adenovirus vectors encoding modified GPs. *PLoS Medicine* 3, e177.

Swanepoel, R., Leman, P.A., Burt, F.J., Zachariades, N.A., Braack, L.E., Ksiazek, T.G., Rollin, P.E., Zaki, S.R. and Peters, C.J. (1996) Experimental inoculation of plants and animals with Ebola virus. *Emerging Infectious Diseases* 2, 321–325.

Swanepoel, R., Smit, S.B., Rollin, P.E., Formenty, P., Leman, P.A., Kemp, A., Burt, F.J., Grobbelaar, A.A., Croft, J., Bausch, D.G., Zeller, H., Leirs, H., Braack, L.E., Libande, M.L., Zaki, S., Nichol, S.T., Ksiazek, T.G. and Paweska, J.T. (2007) Studies of reservoir hosts for Marburg virus. *Emerging Infectious Diseases* 13, 1847–1851.

Swiecki, M. and Colonna, M. (2011) Type I interferons: diversity of sources, production pathways and effects on immune responses. *Current Opinion in Virology* 1, 463–475.

Timen, A., Koopmans, M.P.G., Vossen, A.C.T.M., van Doornum, G.J.J., Günther, S., van den Berkmortel, F., Verduin, K.M., Dittrich, S., Emmerich, P., Osterhaus, A.D.M., van Dissel, J.T. and Coutinho, R.A. (2009) Response to imported case of Marburg hemorrhagic fever, the Netherlands. *Emerging Infectious Diseases* 15, 1171–1175.

Towner, J.S., Khristova, M.L., Sealy, T.K., Vincent, M.J., Erickson, B.R., Bawiec, D.A., Hartman, A.L., Comer, J.A., Zaki, S.R., Stroher, U., Gomes da Silva, F., del Castillo, F., Rollin, P.E., Ksiazek, T.G. and Nichol, S.T. (2006) Marburgvirus genomics and association with a large hemorrhagic fever outbreak in Angola. *Journal of Virology* 80, 6497–6516.

Towner, J.S., Pourrut, X., Albarino, C.G., Nkogue, C.N., Bird, B.H., Grard, G., Ksiazek, T.G., Gonzalez, J.P., Nichol, S.T. and Leroy, E.M. (2007) Marburg virus infection detected in a common African bat. *PLoS One* 2, e764.

Towner, J.S., Amman, B.R., Sealy, T.K., Carroll, S.A.R., Comer, J.A., Kemp, A., Swanepoel, R., Paddock, C.D., Balinandi, S., Khristova, M.L., Formenty, P.B.H., Albarino, C.G., Miller, D.M., Reed, Z.D., Kayiwa, J.T., Mills, J.N., Cannon, D.L., Greer, P.W., Byaruhanga, E., Farnon, E.C., Atimnedi, P., Okware, S., Katongole-Mbidde, E., Downing, R., Tappero, J.W., Zaki, S.R., Ksiazek, T.G., Nichol, S.T. and Rollin, P.E. (2009) Isolation of genetically diverse Marburg viruses from Egyptian fruit bats. *PLoS Pathogen* 5, e100000536.

Valmas, C., Grosch, M.N., Schumann, M., Olejnik, J., Martinez, O., Best, S.M., Krahling, V., Basler, C.F. and Mühlberger, E. (2010) Marburg virus evades interferon responses by a mechanism distinct from Ebola virus. *PLoS Pathogen* 6, e1000721.

Warfield, K. L., Swenson, D.L., Olinger, G.G., Kalina, W.V., Aman, M.J. and Bavari, S. (2007) Ebola virus-like particle-based vaccine protects nonhuman primates against lethal Ebola virus challenge. *Journal of Infectious Diseases* 196, S430–437.

Weber, F., Kochs, G. and Haller, O. (2004) Inverse interference: how viruses fight the interferon system. *Viral Immunology* 17, 498–515.

WHO (1976) Viral haemorrhagic fever. *Bulletin of the World Health Organization* 42, 327.

WHO (1992) Viral haemorrhagic fever in imported monkeys. *Weekly Epidemiological Record* 67, 142.

WHO (2005) Marburg haemorrhagic fever, Angola – update. *Weekly Epidemiological Record* 80, 298.

WHO (2007) Outbreak of Marburg haemorrhagic fever: Uganda, June–August 2007. *Weekly Epidemiological Record* 82, 381–384.

Zlotnik, I. (1969) Marburg agent disease: pathology. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 63, 310–327.

16 Phocine Distemper Virus

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16.1 Introduction

A mass mortality exceeding 23,000 harbour seals (*Phoca vitulina*) in the North Sea during 1988 led to a scramble to identify the causative agent, eventually pointing to a novel morbillivirus, closely related to but distinct from canine distemper virus (CDV) (Osterhaus and Vedder, 1988; Dietz *et al.*, 1989a, 1989b; Cosby *et al.*, 1988; Curran *et al.*, 1990; Heidejorgensen *et al.*, 1992). This previously unknown virus, subsequently named phocine distemper virus (PDV), has since been the focus of extensive research, providing an important model for emerging infectious diseases in marine systems (Hall *et al.*, 2006). Since 1988 it has been recognized that morbilliviruses are important causes of infectious disease mortality in marine mammals around the world, with CDV and PDV epizootics occurring in a range of pinniped species (Osterhaus *et al.*, 1989a, 1992; Kennedy, 1998; Barrett, 1999; Kennedy *et al.*, 2000), including a second major PDV epizootic in European harbour seals that killed 30,000 animals in 2002 (Harkonen *et al.*, 2006), and mortalities in cetaceans due to dolphin and porpoise morbillivirus (CMV, PMV) (Kennedy *et al.*, 1992; Barrett *et al.*, 1993) and sirenians (Duignan *et al.*, 1995a). In this chapter, I briefly review the structure, genetics and biology of PDV.

16.2 Virus Structure, Diversity, Taxonomy and Molecular Interactions with Hosts

The phocine distemper virus particle has an envelope, and the helical nucleocapsid core contains a single-stranded, non-segmented negative-sense 15.6 kilobase pair RNA genome. The genome has six transcriptional units/genes, aligned with the structure observed for other morbilliviruses, and these are annotated as per the structural proteins in other congeneric viruses: 3′ – N gene (1683 base pairs), P gene (1644 base pairs), M gene (1683 base pairs), F gene (2206 base pairs), H gene (1952 base pairs), L gene (~8900 base pairs) – 5′ (Curran *et al.*, 1990, 1992; Kovamees *et al.*, 1991; Rima *et al.*, 1992; Blixenkrone-Moller, 1993).

Eight proteins are encoded among the six genes: nucleoscapsid (N, 523 amino acids); phosphoprotein (P, 507 amino acids); matrix protein (M, 335 amino acids); fusion glycoprotein (F, 537 amino acids); haemagglutinin/neuraminidase glycoprotein (H, 607 amino acids); and large RNA-dependent polymerase (L, 2184 amino acids). The P gene of PDV additionally codes for two further distinct non-structural proteins, V (299 amino acids) and C (174 amino acids). Together the M, F and H proteins are associated with the viral envelope (Cosby *et al.*,

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1988; Curran *et al.*, 1992; Rima *et al.*, 1992; Blixenkrone-Moller, 1993).

Population-level studies of genetic diversity in PDV are relatively limited, but assessments have been made between small numbers of isolates obtained during mortalities in seals and for an isolate derived from Alaskan sea otters (*Enhydra lutris*), using a mixture of different gene fragments (typically P, M, F or H genes). In each case only minor differences have been observed between strains. Analysis of H gene sequences by Nielsen *et al.* (2009), for wild 1988 and 2002 North Sea epizootic isolates (i.e. not passaged through ferrets as in some previous studies), showed 14 nucleotide differences between the two isolates, resulting in eight amino acid differences (98.7% amino acid identity). Sequencing of an isolate derived from a harbour seal stranded during a mortality event in Maine on the north-eastern coast of the USA in 2006 (Earle *et al.*, 2011) showed 11 amino acid differences in the H gene between it and the 2002 North Sea isolate, placing it closer to the 1988 strain. By comparison, H gene divergence between PDV and CDV is 29% and 25–26% at the nucleotide and amino acid level respectively. Phylogenetic analyses showed the 2002 North Sea strain to be more closely related to a putative ancestral PDV sequence than the 1988 North Sea isolate, suggesting the 2002 epizootic derived from a reintroduction of the virus to the North Sea population rather than arising from the 1988 strain, which persisted either in the seal population or a terrestrial reservoir (Nielsen *et al.*, 2009).

More limited sequence data for the P gene exist for additional strains. Comparisons of sequences from harbour seal outbreaks, with isolates from harp seal (*Phoca groenlandia*; Gulf of St Lawrence 1991), hooded seal (*Cystophora cristata*; New Jersey, USA 1998) and sea otters (Alaska 2004–2008), place the 2006 Maine isolate with the 1988 North Sea strains, while the 2006 sea otter, 1991 harp seal and 1998 hooded seal isolates were identical to the P gene sequence of the 2002 North Sea PDV isolate (Goldstein *et al.*, 2009; Earle *et al.*, 2011). Overall this suggests the circulation of multiple closely related PDV strains in north Atlantic and Arctic pinnipeds. Within the overall phylogeny of morbilli and paramyxoviruses, all PDV strains are consistently placed as a monophyletic sister clade to CDV across all genes (McCarthy and Goodman, 2010); recent evidence points to bats as the origin of the CDV/PDV group (Drexler *et al.*, 2012).

In common with other morbilliviruses, it is assumed that PDV uses the lymphocyte-associated receptor CD150 (also known as signal lymphocyte activation protein, SLAM) as a primary receptor and CD46 (membrane co-factor protein, MCP) as a secondary receptor (Tatsuo *et al.*, 2001; Tatsuo and Yanagi, 2002). Recently McCarthy *et al.* (2011) confirmed expression of SLAM and CD46 in harbour seals. The known infection pathways for morbilliviruses also suggest potential interactions with Toll-like receptors, interferon gamma (IFNG), interleukin-4 (IL4), IL8, IL10 and the vitamin A receptor (RARa) (Hall *et al.*, 2006; McCarthy *et al.*, 2011). Experimental studies using CDV in ferret models suggests that the H protein is a key determinant of virus interactions with SLAM, and that variation in the H protein may influence tissue tropism (Seki *et al.*, 2003; Vongpunsawad *et al.*, 2004; von Messling *et al.*, 2005). Molecular evolution studies of CDV isolates from non-dog carnivores indicate that variation at a small number of key H protein residues involved in binding to SLAM may drive CDV adaptation to new hosts (McCarthy *et al.*, 2007). Nielsen *et al.* (2009) reported that two clusters of H amino acid residues, positions 526–529 and 547–548 and amino acid 552 that have been implicated in the CDV as playing a role in SLAM binding were highly conserved, possibly reflecting overlapping host ranges between PDV and CDV (von Messling *et al.*, 2005).

16.3 Clinical and Pathological Impacts of PDV

Confirmed large-scale mortalities due to PDV have only been observed in European

harbour seals during the 1988 and 2002 epizootics. In these outbreaks the clinical signs observed were similar to other morbilliviruses, including respiratory problems, fever, oculonasal discharge, conjunctivitis, ophthalmitis, keratitis, coughing, dyspnoea, diarrhoea, abortion, increased buoyancy and an inability to dive (Bergman *et al.*, 1990; Kennedy, 1990; Harkonen *et al.*, 2006). Morbilliviruses are often immunosuppressive and in harbour seals death was frequently caused by secondary bacterial infections by agents such as *Bordetella bronchiseptica* (Muller *et al.*, 2004). Pathological findings included interstitial and purulent pneumonias with alveolar and interstitial emphysema and generalized lymphodepletion (Kennedy, 1990). Incubation time ranged between 5 and 12 days and changes in neutralizing serum antibody titres in seals were apparent from 16 days post infection (Harder *et al.*, 1990). Diagnosis at necropsy is usually based on a combination of microscopic evidence showing generalized lymphoid depletion and acute interstitial pneumonia, together with molecular evidence of infection with PDV from reverse transcriptase polymerase chain reaction (rtPCR) amplification of PDV sequences or detection of PDV-specific antigens via immunohistochemistry.

For sea otters necropsied during the Alaskan mortality event, potential secondary bacterial infections of *Streptococcus infantarius* subsp. *coli* (*S. bovis/equinus* complex) causing alvular endocarditis and septicaemia in mature adults were reported in association with PDV infections, suggesting possible immunosuppressive effects similar to those seen in European harbour seals (Goldstein *et al.*, 2009).

Antibodies and nucleic acid sequences for PDV have been detected in numerous species (see below), but without the high levels of mortality reported for European harbour seals. For example, serological evidence demonstrates widespread exposure of European grey seals (*Halichoerus grypus*) to PDV in 1988 and 2002, but there have been no substantiated cases of fatal PDV infections in adults of this species (Hall *et al.*, 2006), suggesting that grey seals may act as asymptomatic carriers of the infection. Similarly, where evidence of PDV infection has been detected in other phocid seals, this is also in the absence of obvious mortality events. This suggests that there are substantial inter-species differences in PDV susceptibility and mortality, but the underlying genetic, environmental and epidemiological causes of these differences remain to be determined.

16.4 PDV Hosts and Global Distribution

After the first identification of PDV in European harbour seals following the 1988 epizootic, numerous surveys for morbilliviruses have been carried out in archived sera and tissue from pinnipeds and potential terrestrial hosts, together with now routine testing of stranded pinnipeds.

No direct evidence has been reported demonstrating the presence of PDV in European seals prior to 1988 (Osterhaus *et al.*, 1988, 1989b; Harwood *et al.*, 1989), although anecdotal historical accounts hint at harbour seals with symptoms consistent with PDV in the Orkneys during the 1930s (Harwood and Hall, 1990). Serological evidence for PDV neutralizing antibodies prior and subsequent to 1988 has been reported in archived sera from Arctic populations of grey seals (Carter *et al.*, 1992; Henderson *et al.*, 1992; Duignan *et al.*, 1995b), harp seals (Dietz *et al.*, 1989a; Henderson *et al.*, 1992; Markussen and Have, 1992; Duignan *et al.*, 1997), hooded seals (Henderson *et al.*, 1992; Duignan *et al.*, 1997), ringed seals (*Phoca hispada*) (Dietz *et al.*, 1989a; Henderson *et al.*, 1992; Duignan *et al.*, 1997), walrus (*Odobenus rosmarus*) (Duignan *et al.*, 1994; Nielsen *et al.*, 2000) and harbour seal populations along the north-eastern coast of the USA (Carter *et al.*, 1992; Duignan *et al.*, 1993, 1995b), indicating that PDV has been endemic within Arctic pinniped populations for a considerable time. Limited surveys of spotted (*Phoca larga*), ribbon (*Phoca fasciata*) and bearded

seals (*Erignathus barbatus*), plus stellar sea lions (*Eumatopius jubatus*), did not show any evidence of exposure (Osterhaus *et al.*, 1988).

Antibody prevalence in European harbour seals after the 1988 and 2002 epizootics showed considerable declines, being detectable only in seals less than 2 months old, or adults that would have survived the epizootic, demonstrating the virus was not circulating in Europe after the outbreaks (Harkonen *et al.*, 2006; Lonergan *et al.*, 2010; Bodewes *et al.*, 2013).

To date no evidence has been presented confirming the presence of PDV in Otarid species, Pacific phocids, or any Antarctic pinnipeds, although a future spread into such populations/species cannot be ruled out. Indeed we may expect to see spread of PDV into the Pacific given the incidence of PDV in Alaskan sea otters, which suggests movement into the north Pacific of a vector capable of transmitting the virus from Arctic seal species, in which the virus is likely to be endemic.

PDV antibodies have been detected in Canadian populations of black (*Ursus americanus*), grizzly (*Ursus arctos horribilis*) and polar bears (*Ursus maritimus*), lynx (*Lynx canadensis*) and wolves (*Canis lupus*) (Philippa *et al.*, 2004). Presumably exposure to PDV came from predation or scavenging on pinnipeds, but it is not known whether PDV can sustain infections in these species. PDV has also been recovered from Alaskan sea otters and can be passaged experimentally in ferrets, suggesting that like CDV it may also be capable of infecting mustelids. However, there is not thought to be a link between terrestrial carnivores and the PDV outbreaks in Europe (Harkonen *et al.*, 2006).

CDV is widely distributed in some phocid seals and walrus and has caused mass mortalities in Caspian seals (*Pusa caspica*) (Kennedy *et al.*, 2000), Baikal seals (*Pusa siberica*) (Osterhaus *et al.*, 1989a), and possibly some Antarctic species (Bengtson *et al.*, 1991). However, these incidents are all epidemiologically independent of PDV (Harkonen *et al*, 2006).

16.5 Epidemiology and Contributing Factors in European PDV Epizootics

The two European PDV epizootics showed interesting epidemiological patterns with considerable geographic variation in mortality among harbour seal populations across Europe. Both started on the small island of Anholt in the central Danish Kattegat, which is one of the few sites in Europe with mixed harbour and grey seal haulouts. The 1988 epizootic began on 12 April, while the 2002 outbreak began 3 weeks later on 4 May. In both events the disease spread rapidly to other seal haulout sites in the North Sea, Baltic and as far as the east coast of Northern Ireland over a period of around 9 months. Typically outbreaks lasted for 6–11 weeks in particular geographic locations. In both epizootics the highest levels of mortality were observed in the southern and eastern parts of the North Sea, ranging from more than 50% in Kattegat, Skagerrak, Baltic, Waddensea and East Anglia in 1988, to as low as 1–13% around Scotland. Several populations showed reduced mortality in 2002 compared with 1988, particularly the Baltic, East Anglia and Scotland, which has been attributed to a later arrival of the virus to these areas in 2002, which may have led to a reduced R_0 due to lower seal densities/contact rates at haulouts later in the year (Harkonen *et al.*, 2006).

Epidemiological models suggest that around 300,000 individuals are needed to maintain morbilliviruses as endemic infections (Swinton *et al.*, 1998). The European harbour seal and grey seal populations are well below this figure, which explains the failure of PDV to persist after introduction in 1988. Indeed the decline in seroprevalence in harbour seals and the interval between the two outbreaks fits well with epidemiological model predictions. The most recent surveys of seroprevalence indicate only 11% of harbour seals in the Waddensea have antibodies to PDV, so there is potential for another epizootic on comparable scales to the previous two in the near future (Bodewes *et al.*, 2013). Recurrent epizootics on the scale previously seen have

the potential to drive declines in populations, potentially causing more than a five-fold increase in quasi-extinction risk (a decline to 10% initial population size within 100 years) for some populations, depending on severity and periodicity (Harding *et al.*, 2002, 2003; Lonergan and Harwood, 2003).

Arctic seal populations are large enough to sustain PDV as an endemic infection, and would therefore appear to be the reservoir for the infections that periodically spread into the European populations. Prior to 1988, European populations were recovering from many decades of hunting and depressed fertility due to organochlorine contamination. The epizootics therefore coincide with populations reaching sizes sufficient to sustain epizootics, and the movement of an infectious vector from the Arctic. Harp seals and grey seals migrating from Arctic waters into the North Sea have both been suggested as vectors that may have initiated the epizootics (Harkonen *et al.*, 2006). While perhaps the most plausible explanation, only circumstantial evidence exists to indicate the origins of the epizootics. Harp seals moved south into the North Sea in large numbers in the winter of 1987–88 (Dietz *et al.*, 1989a; Henderson *et al.*, 1992; Markussen and Have, 1992), while grey seals are known to make long-range movements from high latitudes into the southern North Sea, and share the haulout with harbour seals on Anholt (Harkonen *et al.*, 2006).

The high levels of mortality in European harbour seals relative to other seal species, and the large differences in mortality between different geographic populations of harbour seal in Europe, has stimulated much interest in the factors that might account for these differences (Harwood and Hall, 1990; Hall *et al.*, 2006; Harkonen *et al.*, 2006). Between species variation may stem from genetic differences in virus–host interactions, or the extent of susceptibility to the immunosuppressive effects of organochlorine contaminants. Variation in mortality among European harbour seal populations is at least partly driven by epidemiological factors, such as the timing of arrival of the virus, population density and contact rates (Harkonen *et al.*, 2006). Roles for possible immune suppression by organochlorines, burdens of which can vary between areas, and for genetic differences in susceptibility have also been proposed, but strong empirical support for these factors remains to be identified (Goodman, 1998; Hall *et al.*, 2006; Harkonen *et al.*, 2006; McCarthy *et al.*, 2011).

References

Barrett, T. (1999) Morbillivirus infections, with special emphasis on morbilliviruses of carnivores. *Veterinary Microbiology* 69, 3–13.

Barrett, T., Visser, I.K.G., Mamaev, L., Goatley, L., Vanbressem, M.F. and Osterhaus, A. (1993) Dolphin and porpoise morbilliviruses are genetically distinct from phocine distemper virus. *Virology* 193, 1010–1012.

Bengtson, J.L., Boveng, P., Franzen, U., Have, P., Heidejorgensen, M.P. and Harkonen, T.J. (1991) Antibodies to canine-distemper virus in Antarctic seals. *Marine Mammal Science* 7, 85–87.

Bergman, A., Jarplid, B. and Svensson, B.M. (1990) Pathological findings indicative of distemper in European seals. *Veterinary Microbiology* 23, 331–341.

Blixenkrone-Moller, M. (1993) Biological properties of phocine distemper virus and canine distemper virus. *APMIS Supplementum* 36, 1–51.

Bodewes, R., Morick, D., Van De Bildt, M.W.G., Osinga, N., Rubio Garcia, A., Sanchez Contreras, G.J., Smits, S.L., Reperant, L.A.P., Kuiken, T. and Osterhaus, A.D.M.E. (2013) Prevalence of phocine distemper virus specific antibodies: bracing for the next seal epizootic in north-western Europe. *Emerging Microbes & Infections* 2, http://dx.doi.org/10.1038/emi.2013.2

Carter, S.D., Hughes, D.E., Taylor, V.J. and Bell, S.C. (1992) Immune-responses in common and gray seals during the seal epizootic. *Science of the Total Environment* 115, 83–91.

Cosby, S.L., McQuaid, S., Duffy, N., Lyons, C., Rima, B.K., Allan, G.M., McCullough, S.J., Kennedy, S., Smyth, J.A., Mcneilly, F., Craig, C. and Orveli, C. (1988) Characterization of a seal morbillivirus. *Nature* 336, 115–116.

Curran, M.D., Oloan, D., Rima, B.K. and Kennedy, S. (1990) Nucleotide-sequence analysis of phocine distemper virus reveals its distinctness from canine-distemper virus. *Veterinary Record* 127, 430–431.

Curran, M.D., Oloan, D., Kennedy, S. and Rima, B.K. (1992) Molecular characterization of phocine distemper virus – gene order and sequence of the gene encoding the attachment (H) protein. *Journal of General Virology* 73, 1189–1194.

Dietz, R., Ansen, C.T., Have, P. and Heidejorgensen, M.P. (1989a) Clue to seal epizootic. *Nature* 338, 627–627.

Dietz, R., Heidejorgensen, M.P. and Harkonen, T. (1989b) Mass deaths of harbor seals (*Phoca vitulina*) in Europe. *Ambio* 18, 258–264.

Drexler, J.F., Corman, V.M., Muller, M.A., Maganga, G.D., Vallo, P., Binger, T., Gloza-Rausch, F., Rasche, A., Yordanov, S., Seebens, A., Oppong, S., Sarkodie, Y.A., Pongombo, C., Lukashev, A.N., Schmidt-Chanasit, J., Stocker, A., Carneiro, A.J.B., Erbar, S., Maisner, A., Fronhoffs, F., Buettner, R., Kalko, E.K.V., Kruppa, T., Franke, C.R., Kallies, R., Yandoko, E.R.N., Herrler, G., Reusken, C., Hassanin, A., Kruger, D.H., Matthee, S., Ulrich, R.G., Leroy, E.M. and Drosten, C. (2012) Bats host major mammalian paramyxoviruses. *Nature Communications* 3.

Duignan, P.J., Sadove, S., Saliki, J.T. and Geraci, J.R. (1993) Phocine distemper in harbor seals (*Phoca vitulina*) from Long-Island, New York. *Journal of Wildlife Diseases* 29, 465–469.

Duignan, P.J., Saliki, J.T., Staubin, D.J., House, J.A. and Geraci, J.R. (1994) Neutralizing antibodies to phocine distemper virus in Atlantic walruses (*Odobenus rosmarus rosmarus*) from Arctic Canada. *Journal of Wildlife Diseases* 30, 90–94.

Duignan, P.J., House, C., Walsh, M.T., Campbell, T., Bossart, G.D., Duffy, N., Fernandes, P.J., Rima, B.K., Wright, S. and Geraci, J.R. (1995a) Morbillivirus infection in manatees. *Marine Mammal Science* 11, 441–451.

Duignan, P.J., Saliki, J.T., Aubin, D.J.S., Early, G., Sadove, S., House, J.A., Kovacs, K. and Geraci, J.R. (1995b) Epizootiology of morbillivirus infection in North American harbor seals (*Phoca vitulina*) and gray seals (*Halichoerus grypus*). *Journal of Wildlife Diseases* 31, 491–501.

Duignan, P.J., Nielsen, O., House, C., Kovacs, K.M., Duffy, N., Early, G., Sadove, S., Staubin, D.J., Rima, B.K. and Geraci, J.R. (1997) Epizootiology of morbillivirus infection in harp, hooded, and ringed seals from the Canadian Arctic and western Atlantic. *Journal of Wildlife Diseases* 33, 7–19.

Earle, J.A.P., Melia, M.M., Doherty, N.V., Nielsen, O. and Cosby, S.L. (2011) Phocine distemper virus in seals, east coast, United States, 2006. *Emerging Infectious Diseases* 17, 215–220.

Goldstein, T., Mazet, J.A.K., Gill, V.A., Doroff, A.M., Burek, K.A. and Hammond, J.A. (2009) Phocine distemper virus in northern sea otters in the Pacific Ocean, Alaska, USA. *Emerging Infectious Diseases* 15, 925–927.

Goodman, S.J. (1998) Patterns of extensive genetic differentiation and variation among European harbor seals (*Phoca vitulina vitulina*) revealed using microsatellite DNA polymorphisms. *Molecular Biology and Evolution* 15, 104–118.

Hall, A.J., Jepson, P.D., Goodman, S.J. and Harkonen, T. (2006) Phocine distemper virus in the North and European Seas – data and models, nature and nurture. *Biological Conservation* 131, 221–229.

Harder, T., Willhaus, T., Frey, H.R. and Liess, B. (1990) Morbillivirus infections of seals during the 1988 epidemic in the Bay of Heligoland. 3. Transmission studies of cell culture-propagated phocine distemper virus in harbor seals (*Phoca vitulina*) and a gray seal (*Halichoerus grypus*) – clinical, virological and serological results. *Journal of Veterinary Medicine Series B – Zentralblatt Fur Veterinarmedizin Reihe B –Infectious Diseases and Veterinary Public Health* 37, 641–650.

Harding, K.C., Harkonen, T. and Caswell, H. (2002) The 2002 European seal plague: epidemiology and population consequences. *Ecology Letters* 5, 727–732.

Harding, K.C., Harkonen, T. and Pineda, J. (2003) Estimating quasi-extinction risk of European harbour seals: reply to Lonergan & Harwood (2003). *Ecology Letters* 6, 894–897.

Harkonen, T., Dietz, R., Reijnders, P., Teilmann, J., Harding, K., Hall, A., Brasseur, S., Siebert, U., Goodman, S.J., Jepson, P.D., Rasmussen, T.D. and Thompson, P. (2006) The 1988 and 2002 phocine distemper virus epidemics in European harbour seals. *Diseases of Aquatic Organisms* 68, 115–130.

Harwood, J. and Hall, A. (1990) Mass mortality in marine mammals – its implications for population-dynamics and genetics. *Trends in Ecology & Evolution* 5, 254–257.

Harwood, J., Carter, S.D., Hughes, D.E., Bell, S.C., Baker, J.R. and Cornwell, H.J.C. (1989) Seal disease predictions. *Nature* 339, 670–670.

Heidejorgensen, M.P., Harkonen, T., Dietz, R. and Thompson, P.M. (1992) Retrospective of the 1988 European seal epizootic. *Diseases of Aquatic Organisms* 13, 37–62.

Henderson, G., Trudgett, A., Lyons, C. and Ronald, K. (1992) Demonstration of antibodies in archival sera from Canadian seals reactive with a European isolate of phocine distemper virus. *Science of the Total Environment* 115, 93–98.

Kennedy, S. (1990) A review of the 1988 European seal morbillivirus epizootic. *Veterinary Record* 127, 563–567.

Kennedy, S. (1998) Morbillivirus infections in aquatic mammals. *Journal of Comparative Pathology* 119, 201–225.

Kennedy, S., Kuiken, T., Ross, H.M., Mcaliskey, M., Moffett, D., McNiven, C.M. and Carole, M. (1992) Morbillivirus infection in 2 common porpoises (*Phocoena phocoena*) from the coasts of England and Scotland. *Veterinary Record* 131, 286–290.

Kennedy, S., Kuiken, T., Jepson, P.D., Deaville, R., Forsyth, M., Barrett, T., Van De Bildt, M.W.G., Osterhaus, A., Eybatov, T., Duck, C., Kydyrmanov, A., Mitrofanov, I. and Wilson, S. (2000) Mass die-off of Caspian seals caused by canine distemper virus. *Emerging Infectious Diseases* 6, 637–639.

Kovamees, J., Blixenkronemoller, M., Sharma, B., Orvell, C. and Norrby, E. (1991) The nucleotide-sequence and deduced amino-acid-composition of the hemagglutinin and fusion proteins of the morbillivirus phocid distemper virus. *Journal of General Virology* 72, 2959–2966.

Lonergan, M. and Harwood, J. (2003) The potential effects of repeated outbreaks of phocine distemper among harbour seals: a response to Harding *et al*. (2002). *Ecology Letters* 6, 889–893.

Lonergan, M., Hall, A., Thompson, H., Thompson, P.M., Pomeroy, P. and Harwood, J. (2010) Comparison of the 1988 and 2002 phocine distemper epizootics in British harbour seal *Phoca vitulina* populations. *Diseases of Aquatic Organisms* 88, 183–188.

Markussen, N.H. and Have, P. (1992) Phocine distemper virus-infection in harp seals (*Phoca groenlandica*). *Marine Mammal Science* 8, 19–26.

McCarthy, A.J. and Goodman, S.J. (2010) Reassessing conflicting evolutionary histories of the Paramyxoviridae and the origins of respiroviruses with Bayesian multigene phylogenies. *Infection Genetics and Evolution* 10, 97–107.

McCarthy, A.J., Shaw, M.-A. and Goodman, S.J. (2007) Pathogen evolution and disease emergence in carnivores. *Proceedings of the Royal Society B – Biological Sciences* 274, 3165–3174.

McCarthy, A.J., Shaw, M.-A., Jepson, P.D., Brasseur, S.M.J.M., Reijnders, P.J.H. and Goodman, S.J. (2011) Variation in European harbour seal immune response genes and susceptibility to phocine distemper virus (PDV). *Infection Genetics and Evolution* 11, 1616–1623.

Muller, G., Wohlsein, P., Beineke, A., Haas, L., Greiser-Wilke, I., Siebert, U., Fonfara, S., Harder, T., Stede, M., Gruber, A.D. and Baumgartner, W. (2004) Phocine distemper in German seals, 2002. *Emerging Infectious Diseases* 10, 723–725.

Nielsen, L., Arctander, P., Jensen, T.H., Dietz, H.-H., Hammer, A.S., Banyard, A.C., Barrett, T. and Blixenkrone-Moller, M. (2009) Genetic diversity and phylogenetic analysis of the attachment glycoprotein of phocine distemper viruses of the 2002 and 1988 epizootics. *Virus Research* 144, 323–328.

Nielsen, O., Stewart, R.E.A., Measures, L., Duignan, P. and House, C. (2000) A morbillivirus antibody survey of Atlantic walrus, Narwhal and Beluga in Canada. *Journal of Wildlife Diseases* 36, 508–517.

Osterhaus, A. and Vedder, E.J. (1988) Identification of virus causing recent seal deaths. *Nature* 335, 20.

Osterhaus, A., Groen, J., Devries, P., UytdeHaag, F., Klingeborn, B. and Zarnke, R. (1988) Canine-distemper virus in seals. *Nature* 335, 403–404.

Osterhaus, A., Groen, J., UytdeHaag, F., Vanderbildt, M.W.G., Visser, I.K.G., Bergman, A. and Klingeborn, B. (1989a) Distemper virus in Baikal seals. *Nature* 338, 209–210.

Osterhaus, A., Groen, J., UytdeHaag, F., Visser, I.K.G., Vedder, E.J., Crowther, J. and Bostock, C.J. (1989b) Morbillivirus infections in European seals before 1988. *Veterinary Record* 125, 326–326.

Osterhaus, A., Visser, I.K.G., Deswart, R.L., Vanbressem, M.F., Vandebildt, M.W.G., Orvell, C., Barrett, T. and Raga, J.A. (1992) Morbillivirus threat to Mediterranean monk seals. *Veterinary Record* 130, 141–142.

Philippa, J.D.W., Leighton, F.A., Daoust, P.Y., Nielsen, O., Pagliarulo, M., Schwantje, H., Shury, T., Van Herwijnen, R., Martina, B.E.E., Kuiken, I., Van De Bildt, M.W.G. and Osterhaus, A. (2004) Antibodies to selected pathogens in free-ranging terrestrial carnivores and marine mammals in Canada. *Veterinary Record* 155, 135–140.

Rima, B.K., Curran, M.D. and Kennedy, S. (1992) Phocine distemper virus, the agent responsible for the 1988 mass mortality of seals. *Science of the Total Environment* 115, 45–55.

Seki, F., Ono, N., Yamaguchi, R. and Yanagi, Y. (2003) Efficient isolation of wild strains of canine distemper virus in Vero cells expressing canine SLAM (CD150) and their adaptability to marmoset B95a cells. *Journal of Virology* 77, 9943–9950.

Swinton, J., Harwood, J., Grenfell, B.T. and Gilligan, C.A. (1998) Persistence thresholds for phocine distemper virus infection in harbour seal *Phoca vitulina* metapopulations. *Journal of Animal Ecology* 67, 54–68.

Tatsuo, H. and Yanagi, Y. (2002) The morbillivirus receptor SLAM (CD150). *Microbiology and Immunology* 46, 135–142.

Tatsuo, H., Ono, N. and Yanagi, Y. (2001) Morbilliviruses use signaling lymphocyte activation molecules (CD150) as cellular receptors. *Journal of Virology* 75, 5842–5850.

Von Messling, V., Oezguen, N., Zheng, Q., Vongpunsawad, S., Braun, W. and Cattaneo, R. (2005) Nearby clusters of hemagglutinin residues sustain SLAM-dependent canine distemper virus entry in peripheral blood mononuclear cells. *Journal of Virology* 79, 5857–5862.

Vongpunsawad, S., Oezgun, N., Braun, W. and Cattaneo, R. (2004) Selectively receptor-blind measles viruses: identification of residues necessary for SLAM- or CD46-induced fusion and their localization on a new hemagglutinin structural model. *Journal of Virology* 78, 302–313.

17 Morbilliviruses in Sea Mammals

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17.1 Introduction

In the last three decades new morbilliviruses have emerged as a serious threat to marine mammal populations. Recognition of morbilliviruses as infectious agents in marine mammals dates from the late 1980s, when two nearby simultaneous distemper epizootics occurred in the Baikal Lake, affecting Baikal seals (*Phoca sibirica*) (Grachev *et al.*, 1989; Osterhaus *et al.*, 1989a, 1989b; Visser *et al.*, 1990), and in the North and Baltic Seas, affecting harbour seals (*Phoca vitulina*) and grey seals (*Halichoerus grypus*). Canine distemper virus (CDV) caused the epizootic at Baikal Lake, whereas the seals in northern Europe were affected by a previously unknown morbillivirus, different from CDV, called phocine distemper virus (PDV) (Cosby *et al.*, 1988; Kennedy *et al.*, 1988a; Visser *et al.*, 1990). The next known morbillivirus epizootic in marine mammals affected Mediterranean striped dolphins (*Stenella coeruleoalba*) and common dolphins in the Black Sea (*Delphinus delphis ponticus*) from 1990 to 1994 (Domingo *et al.*, 1990; Birkun *et al.*, 1999). This mass mortality was caused by a new member of the morbillivirus genus, called dolphin morbillivirus (DMV) (Domingo *et al.*, 1990). A previous epizootic affecting bottlenose dolphins (*Tursiops truncatus*) on the east coast of the USA in 1987–1988 (Geraci, 1989) was re-evaluated, and it was concluded that a morbillivirus closely related to DMV had a primary role in the die-off (Lipscomb *et al.*, 1994, 1996). The identification of DMV drew attention to a morbillivirus previously found in six harbour porpoises (*Phocoena phocoena*) in the Irish Sea in 1988 (Kennedy *et al.*, 1988b, 1991). The virus was characterized and proposed as a new species of the genus morbillivirus, called porpoise morbillivirus (PMV), closely related to DMV, distinct from other terrestrial morbilliviruses and also from PDV (McCullough *et al.*, 1991; Trudgett *et al.*, 1991). A lethal morbilliviral infection in long-finned pilot whales (*Globicephala melas*) was reported on the New Jersey coast (Taubenberger *et al.*, 2000), caused by another phylogenetically related but distinct morbillivirus, termed pilot whale morbillivirus (PWMV). In addition to epizootic outbreaks, sporadic cases of systemic morbilliviral disease have been reported in both seals and cetaceans (Daoust *et al.*, 1993; Duignan *et al.*, 1993; Jauniaux *et al.*, 2000; Yang *et al.*, 2006; Stone *et al.*, 2011; Mazzariol *et al.*, 2012), and unusual forms of localized central nervous system (CNS) infection have been described in the striped dolphin (Domingo *et al.*, 1995; Soto *et al.*, 2011a).

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Sequences of the different genes of a dolphin morbillivirus, as well as the genome termini and the trailer and leader sequences, have been determined (Blixenkrone-Møller *et al.*, 1994, 1996; Bolt *et al.*, 1995; Rima *et al.*, 2005), and the complete sequence (accession number AJ608288) compared to that of other morbillivirus genomes. The organization of the marine mammal morbilliviruses genome, a single-stranded negative-sense RNA genome of about 16 kb in length, appears to be similar to that of morbilliviruses of terrestrial mammals (Rima *et al.*, 2005). As in other morbilliviruses, the CeMV encodes six transcription units for the six structural proteins, the nucleocapsid (N), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin (H) and the RNA-dependent RNA polymerase (L). Two additional proteins (C and V) are translated from the P gene by alternative start codon recognition. These transcription units are flanked by untranslated regions at the genome termini, known as the genome and antigenome promoters (Rima *et al.*, 2005; Banyard *et al.*, 2008). According to the general organization of all morbilliviruses, the length of the CeMV genome (and probably also that of PDV) is a multiple of six (a nucleocapsid molecule is surrounded by six nucleotides). In morbillivirus genomes, the phase of reading of the six transcripts is in a conserved phase (Tapparel *et al.*, 1998), and with the exception of the F transcripts, the phase is strictly conserved (Rima *et al.*, 2005).

The new morbilliviruses in marine mammals, considered by some to be four different species (Taubenberger *et al.*, 2000; Van de Bildt *et al.*, 2005), are classified today by the International Committee on Taxonomy of Viruses (ICTV, 2011) in two species: cetacean morbillivirus (CeMV, including the strains DMV, PMV and PWMV) and PDV. This terminology will be used throughout this chapter.

17.2 Epidemiology

Morbilliviruses are distributed in marine mammals worldwide, and the number of infected pinniped and cetacean species is high (Barrett *et al.*, 1995; Kennedy, 1998; Van Bressem *et al.*, 1999, 2001). Like other morbilliviruses in terrestrial mammals, they are capable of causing epizootics, with high morbidity and mortality, when entering into a naïve population (Kennedy, 1998). Mass mortalities in marine mammals where morbilliviruses have been demonstrated as the causative agent are shown in Table 17.1. Although the first documented epizootic was recorded in 1987 (Lipscomb *et al.*, 1994), serological evidence of morbillivirus infection in marine mammal populations dates from 1973 (Henderson *et al.*, 1992). In addition, single lethal cases or discrete mortality events related to morbillivirus infection have been detected worldwide, for example, in seals from Canada and the USA (Daoust *et al.*, 1993; Duignan *et al.*, 1993; Earle *et al.*, 2011), in harbour porpoises (*Phocoena phocoena*) from England and Scotland (Kennedy *et al.*, 1992) and in bottlenose dolphins from Australia (Stone *et al.*, 2011). Collectively, these data point to the enzootic circulation of morbilliviruses in many pinniped and cetacean species (Van Bressem *et al.*, 2001; Kennedy, 1998).

Susceptibility to morbillivirus in different marine mammal species, population density and animal migratory movements may influence circulation of morbillivirus in pinniped and cetacean populations (Duignan *et al.*, 1995; Kennedy, 1998). Thus, harp seals (*Phoca groenlandica*) are likely a PDV reservoir, and their southward migratory movements may have precipitated the 1988 epizootic in harbour and grey seals in northern Europe (Dietz *et al.*, 1989; Markussen and Have, 1992). Similarly, pilot whales have been incriminated in the introduction into the Mediterranean Sea of CeMV, responsible for two mass mortalities in Mediterranean dolphins (Kennedy, 1998; Fernández *et al.*, 2008). The second CeMV epizootic of 2006 extended from the strait of Gibraltar eastward through the Mediterranean Sea (Raga *et al.*, 2008). In this event the mortality in dolphins was preceded by deaths in pilot whales (Fernández *et al.*, 2008). The pilot whale

Table 17.1. Morbillivirus epizootics in marine mammals.

Place	Species	Date	Virus	Mortality	References
USA, east coast	*Tursiops truncatus*	1987–1988	PMV/DMV	>742	Lipscomb *et al.* (1994); Taubenberger *et al.* (1996)
Lake Baikal	*Phoca sibirica*	1987	CDV	2000	Grachev *et al.* (1989); Likhoshway *et al.* (1989); Osterhaus *et al.* (1989a)
Northern European coast	*Phoca vitulina* *Halichoerus grypus*	1988	PDV	18,000 300	Harwood *et al.* (1989); Kennedy *et al.* (1988a, 1989); Osterhaus and Vedder (1988)
Irish Sea	*Phocoena phocoena*	1988	PMV	6	Kennedy *et al.* (1988b, 1991)
Mediterranean Sea	*Stenella coeruleoalba* *Delphinus delphis ponticus*	1990–1994	DMV	>4000 47	Domingo *et al.* (1992); Duignan *et al.* (1992); Birkun *et al.* (1999)
Gulf of Mexico	*Tursiops truncatus*	1993–1994	PMV	200	Lipscomb *et al.* (1996); Krafft *et al.* (1995); Taubenberger *et al.* (1996)
Caspian Sea	*Phoca caspica*	1997	CDV	10-fold greater than normal mortality	Forsyth *et al.* (1998)
Mediterranean Sea	*Monachus monachus*	1997	MSMV?[a]	Nearly 300	Osterhaus *et al.* (1997, 1998); Van de Bildt *et al.* (2000, 2001)
Caspian Sea	*Phoca caspica*	2000	CDV	10,000	Kennedy *et al.* (2000); Kuiken *et al.* (2006)
Northern European coast	*Phoca vitulina; Halichoerus grypus*	2002	PDV	21,700	Müller *et al.* (2004, 2008)
Mediterranean Sea	*Stenella coeruleoalba* *Globicephala melas*	2006–2007	DMV	Hundreds 51	Raga *et al.* (2008); Fernández *et al.* (2008); Soto *et al.* (2011b); Gauffier (2008)

[a] MSMV, Monk seal morbillivirus, a new morbillivirus related to CeMV, was detected in seal tissues from the die-off, and has been presumptively implicated in this epizootic, although other causes of this mortality have been proposed (Hernández *et al.*, 1998).

population resident in the strait of Gibraltar, which is subjected to intensive monitoring and photo identification, suffered between summer 2006 and summer 2007. In this period, the survival rate was reduced by 21.2%, which increased mortality from 51–52 deaths to 77–78 deaths. This increase in mortality was attributed to CeMV (Gauffier, 2008). The re-emergence of morbillivirus epizootics in marine mammals has been described on several occasions, for example in Mediterranean dolphins and northern European seals. Factors leading to re-emergence of mass mortalities in previously

affected areas are poorly understood. A build-up of susceptible individuals is probably required for the emergence of a new epizootic. It is conceivable that juvenile animals are affected once they lose maternal immunity (Ross *et al.*, 1994). The available data regarding northern seal populations points to this possibility (Jensen *et al.*, 2002). A similar situation has been observed for CeMV in the Mediterranean Sea after the 1990–1994 epizootic. A review of archive material from necropsies of striped dolphins performed during the inter-epizootic period did not find evidence of CeMV-induced lesions or antigens, thus supporting the view that CeMV had apparently not circulated in that population (Soto *et al.*, 2011b). Also, in limited serological studies, the proportion of susceptible individuals in the population had increased (Van Bressem *et al.*, 2001).

Distemper in seals can also be caused by CDV (Grachev *et al.*, 1989; Lyons *et al.*, 1993; Mamaev *et al.*, 1996). The epizootic in Baikal seals in 1987 stressed for the first time the pathogenic potential of CDV in seals (Grachev *et al.*, 1989; Osterhaus *et al.*, 1989a), demonstrating that CDV may also be a threat for other seal species. On the other hand, PDV has been shown experimentally to be infectious for dogs (Jäger *et al.*, 1990), mink (Blixenkrone-Møller *et al.*, 1989) and ferrets (Nielsen *et al.*, 2008). Whether PDV may also infect other terrestrial carnivore species is unknown. The natural host range for CeMV is mainly restricted to cetaceans, although CeMV strains have been detected in seals (Van de Bildt *et al.*, 2000, 2001; Mazzasiol *et al.*, 2013).

Subsequent to both CeMV epizootics in the Mediterranean Sea, unusual cases of CeMV infection affecting only the CNS have been observed in striped dolphins (Domingo *et al.*, 1995; Soto *et al.*, 2011a; Di Guardo *et al.*, 2013). In these cases, infection with CeMV was restricted to the CNS, as showed by immunohistochemistry (IHC) and reverse-transcriptase-polymerase chain reaction (RT-PCR) (Soto *et al.*, 2011a; Di Guardo *et al.*, 2013). It has been suggested that this CNS-localized form in striped dolphins could be similar to a human disease, subacute sclerosing panencephalitis (SSEP) (Domingo *et al.*, 1995; Soto *et al.*, 2011a), which is caused by a replicative defective measles virus (MeV) (Connolly *et al.*, 1967; Garg, 2008). Therefore, these forms would appear to be epidemiologically irrelevant regarding transmission of CeMV in the population. Nevertheless, observed impact on the population through direct mortality could be high in the two or three years after the epizootic wave (Soto *et al.*, 2011a).

17.3 Pathogenesis

Experimental data on pathogenesis of marine morbillivirus infection are scarce, and pathogenesis is mainly deduced from natural disease investigation and from similarities with morbillivirus infections in terrestrial mammals (Kennedy, 1998). Transmission of CeMV and PDV in nature probably occurs by the oronasal route, through direct contact with oculonasal and oral secretions of infected animals, or through airborne droplets at short distance. However, there is no experimental evidence supporting this hypothesis. It is also unknown if infected animals excrete morbillivirus in faeces and urine, as occurs in dogs with distemper. In harbour seals experimentally infected with PDV by the nasal route, no virus could be isolated from faecal samples of affected individuals, although viral isolation also failed from tissue homogenates of seals dying from infection between 11 and 16 days post-infection (Harder *et al.*, 1992). PDV antigen was detected in intestinal lymphoid tissue and in colonic epithelial cells, supporting the possibility of viral secretion in faeces (Harder *et al.*, 1992; Pohlmeyer *et al.*, 1993). Pinnipeds spend part of their biological cycle in terrestrial environments, highly aggregated, and faeces and urine from infected animals might be relevant for transmission. Other routes of infection, such as trans-placental, lactation or venereal routes, could be postulated but there is no direct evidence supporting these possibilities.

As in dogs (Beineke *et al.*, 2009), the tonsils (pharyngeal in seals and laryngeal in dolphins) in marine mammals may constitute

a primary site of replication for morbillivirus (Harder *et al.*, 1992; Soto *et al.*, 2011b). From the primary point of entry the virus may disseminate systemically to other organs. The distribution of virus and lesions follows the same regular pattern in natural cases of disease in the different marine mammal species. Lung, lymphoid organs and the CNS are the main targets (Kennedy *et al.*, 1989; Domingo *et al.*, 1992), regularly showing cellular damage with viral inclusions and viral antigen. The consequence of lymphoid tissue damage is immunosuppression, which facilitates secondary opportunistic infections by fungal agents, *Toxoplasma gondii* and herpesvirus in dolphins (Domingo *et al.*, 1992; Lipscomb *et al.*, 1994, 1996; Schulman *et al.*, 1997; Bellière *et al.*, 2010; Mazzariol *et al.*, 2012; Soto *et al.*, 2012), and by herpesvirus and bacterial in infections in seals (Osterhaus and Vedder, 1988; Gulland *et al.*, 1997; Kennedy, 1998).

The course of PDV infection has been monitored in experimentally infected seals. In one experiment harbour seals were intranasally infected with PDV, and clinical signs, viraemia and immunological response were recorded (Harder *et al.*, 1992). All the infected animals showed fever starting between day 3 and 6 post-inoculation. Afterwards watery diarrhoea, respiratory disease and central nervous signs were observed. Six out of ten animals died within 11–16 days post-infection, despite supportive treatment. The other four seals recovered. Viraemia was cell associated and PDV could be isolated from buffy coat cells starting from day 5 post-infection and lasting until day 17 post-infection or until death of the animal. PDV antigen could be detected in several tissues, including the respiratory and gastrointestinal tracts, CNS and lymphoid organs. PDV neutralizing antibodies were first detected on day 7 post-inoculation, with exponential increase afterwards, reaching a plateau within the fourth week post-infection. The duration of immunity after natural or experimental PDV infection is unknown, but immune seals are protected from clinical disease when experimentally challenged with a moderate dose of PDV (Harder *et al.*, 1990). Pups born from infected mothers show maternally derived antibodies, which decrease progressively with time and are no longer detectable at approximately 10 weeks of age (Harder *et al.*, 1992, 1993).

Other predisposing or triggering factors in marine mammal morbillivirus epizootics have been a matter of intense debate for decades in the scientific community (Van Bressem *et al.*, 2009). Several studies have focused on the influence of xenobiotics on the immune system of cetaceans (Beineke *et al.*, 2010). Some authors have claimed that pollutants, such as polychlorinated biphenyls (PCBs), and starvation were important co-factors in seal and cetacean morbillivirus epizootics (Aguilar and Borrell, 1994; Olsson *et al.*, 1994; de Swart *et al.*, 1995; Kajiwara *et al.*, 2008). In dolphins it has been suggested that PCBs lead to depressed immunocompetence and increased susceptibility to viral infection (Aguilar and Borrell, 1994; Osterhaus *et al.*, 1995). Morbilliviruses are primary pathogenic agents and are able to cause epizootics in their respective hosts without requiring predisposing factors (Appel *et al.*, 1981; O'Shea, 2000). In an experimental study, seals fed with PCB did not show any difference in the outcome and course of PDV infection compared with non-PCB-fed animals (Harder *et al.*, 1992). However, the PCB load of these animals appeared to be lower than the PCB load of free-ranging seals.

In humans and dogs, MeV and CDV are known to cause chronic latent infections, termed respectively subacute sclerosing panencephalitis (SSEP) and old dog encephalitis (ODE) (Axthelm and Krakowka, 1998; Garg, 2008; Headley *et al.*, 2009). In these rare diseases, after an initial systemic infection, virus latently infecting the CNS reactivates several years later, causing a localized, progressive and fatal neurologic disease. It has been suggested that similar chronic latent infections occur in striped dolphins after CeMV epizootics (Domingo *et al.*, 1995; Soto *et al.*, 2011a). In these cases morbillivirus infection was limited to the brain, with absence of lesions and viral antigen in extraneural sites, such as respiratory and lymphoid systems. In SSPE, mutations in the P, M, F and H genes that affect the functionality of viral proteins have been

determined (Cattaneo *et al.*, 1989; Wong *et al.*, 1989), rendering MeV unable to form infective virions. Whether similar changes also occur in CeMV in these striped dolphins remains to be determined.

17.4 Clinical Signs

Clinical signs of morbilliviral infection have been recorded mostly in pinnipeds, but little information is available in cetaceans. Clinical signs of seals affected by PDV are similar to those in dogs infected by CDV (Osterhaus and Vedder, 1988). Weight loss, lethargy, fever, serous or purulent oculonasal secretion, conjunctivitis and dyspnoea have been reported. Other signs also observed are diarrhoea, abortion, subcutaneous emphysema (which increase buoyancy, impairing diving) and increased tolerance to human presence. Neurological signs such as tremor and abnormal posture have been also described (Kennedy, 1998; Philippa *et al.*, 2009). Dermatologic changes, such as alopecia and crusting, have occasionally been reported (Lipscomb *et al.*, 2001).

Affected cetaceans may show poor body condition, lethargy, tremors and abnormal respiratory and cardiac rate. Some animals present disorientation and abnormal swimming with circling (Piza, 1991; M. Domingo, Barcelona-Spain, 2008, personal communication). Dolphins stranded alive with morbilliviral infection died after several hours or a few days with no response to clinical treatments (Piza, 1991).

17.5 Pathology

Morbillivirus-induced lesions are very similar in pinnipeds and cetaceans, and occur principally in lungs, lymphoid tissues and the CNS.

17.5.1 Macroscopic lesions

Poor body condition and an absence of food in stomach are frequently found in affected animals. In lungs, lesions consist of multiple areas of dark-coloured lung tissue, alternating with inflated, pale zones, which are often only evident in severe cases. Normal lungs in cetaceans do not fully collapse when the chest is opened, due to the cartilaginous reinforcement of airways and the presence of smooth muscle sphincters up to the respiratory bronchioles. Nevertheless, most authors describe absence of pulmonary collapse in morbilliviral disease, both in seals and cetaceans. The lung may show areas of consolidation due to suppurative bronchopneumonia, commonly found in seals but not frequently found in cetaceans. Interstitial (interlobular and subpleural) pulmonary emphysema is frequently found in seals, which may extend to the mediastinum and neck fascia and subcutis. Parasitic pneumonia with nodular lesions is usually present. Pulmonary lymph nodes may appear enlarged and oedematous. Erosive stomatitis has been described mainly in cetaceans (Kennedy *et al.*, 1989, 1991; Domingo *et al.*, 1992; Kennedy, 1998; Fernández *et al.*, 2008; Rijks *et al.*, 2008; Philippa *et al.*, 2009). Dermatitis, with alopecia and crusting, has been described in a hooded seal (*Cystophora crystata*) and a harp seal (*Phoca groenlandica*) (Lipscomb *et al.*, 2001). The load of epizoic parasites was increased during epizootics in dolphins due to the reduced mobility caused by the disease (Aznar *et al.*, 2005).

Macroscopic lesions associated with opportunistic fungal infections have been consistently observed in cetaceans during CeMV epizootics. Large focal haemorrhagic-necrotizing mycotic lesions were observed in the lungs and brain (Domingo *et al.*, 1992; Kennedy *et al.*, 1992; Lipscomb *et al.*, 1994; Soto *et al.*, 2011b). Toxoplasma behaves as an opportunistic condition in the western Mediterranean striped dolphin population, with subclinical infection in around 11% of the individuals (Cabezón *et al.*, 2004), and reactivation during CeMV epizootics. Lesions due to *Toxoplasma gondii* appear as small necrotic foci in lymph nodes and lung, but may not be always macroscopically evident (Domingo *et al.*, 1992; Soto *et al.*, 2011b).

17.5.2 Microscopic lesions

Morbillivirus infection in marine mammals usually causes pneumonia, encephalitis and lymphoid necrosis and depletion (Kennedy *et al.*, 1989, 1991; Domingo *et al.*, 1992; Taubenberger *et al.*, 2000; Fernández *et al.*, 2008). This triad of lesions in the lung, brain and lymphoid organs are also highly compatible with morbillivirus infection in terrestrial mammals (Appel *et al.*, 1981; Caswell and Williams, 2007).

Affected animals usually present bronchiolo-interstitial pneumonia, characterized by infection and necrosis of the bronchiolar and alveolar epithelial cells, and exudation of mononuclear leukocytes in the alveolar septa and alveolar spaces. Necrosis of epithelial cells is followed by regeneration of type II pneumocytes and re-epithelization. Multinucleate syncytial cells are frequently observed in bronchiolar and alveolar lumens (see Plate 1). Nuclear and cytoplasmic eosinophilic inclusions are easily recognized in epithelial cells and syncytia (Kennedy *et al.*, 1989, 1991; Baker, 1992; Domingo *et al.*, 1992; Kennedy, 1998; Rijks *et al.*, 2008). Secondary bacterial suppurative bronchopneumonia is a frequent consequence of immunosuppression in seals (Kennedy, 1998).

Generalized lymphocytolysis and lymphoid depletion are prominent findings in seals and cetaceans infected by morbillivirus. Lymph nodes, spleen, thymus and Peyer's Patches are usually affected. In dolphins, the laryngeal tonsil (Cowan and Smith, 1999) also shows similar lesions (Soto *et al.*, 2011b). There is severe loss of lymphocytes and syncytial cells may replace lymphoid tissue. In pinnipeds, unlike in cetaceans, syncytial formation is a rare finding in these tissues. Cell debris and distended blood vessels are visible. Nuclear and cytoplasmic eosinophilic inclusion bodies can also be observed in syncytial or mononuclear cells (Baker, 1992; Domingo *et al.*, 1992; Kennedy, 1998; Jauniaux *et al.*, 2000; Rijks *et al.*, 2008).

Entry of the virus into the CNS induces a non-suppurative meningoencephalitis characterized by neuronal degeneration and necrosis, with perivascular mononuclear inflammatory infiltrates. Neuronal damage is associated with gliosis and formation of neuronophagic nodules. Viral inclusions may be seen in neurons and glial cells. In dolphins, syncytia may be found also in the CNS in low number, but they are rare in seals. Severe patchy demyelinization may be seen in seals (Kennedy *et al.*, 1989, 1991; Domingo *et al.*, 1992; Kennedy, 1998; Fernández *et al.*, 2008; Rijks *et al.*, 2008).

Inflammation and syncytial cells with viral inclusions can be found sporadically in other organs, such as mammary epithelium, hepatic biliary ducts, renal pelvis, urinary bladder, pancreatic ducts and gastrointestinal tract (Domingo *et al.*, 1992; Kennedy, 1998; Jauniaux *et al.*, 2000).

Skin lesions have been associated with morbillivirus infection in seals, with hyperkeratosis, epidermal hyperplasia with formation of syncytial cells, inflammatory infiltrates and viral inclusions (Lipscomb *et al.*, 2001).

Histopathological lesions in the different affected organs are usually associated with the presence of viral antigen that can be revealed by immunohistochemical staining. Syncytial cells, mononuclear leukocytes, pneumocytes (see Plate 2), neurons, glial cells, ependymal cells, keratinocytes and epithelial cells in other organs, such as mammary gland, male reproductive tract and the epithelium overlying the laryngeal tonsil (see Plate 3), show cytoplasmic and nuclear immunostaining (Domingo *et al.*, 1992; Kennedy *et al.*, 1989, 1991; Kennedy, 1998; Fernández *et al.*, 2008).

In dolphins, secondary fungal infections affecting the brain and lungs appear as haemorrhagic-necrotizing pneumonia and encephalitis, with growth of fungal hyphae in the tissues and in vessels, causing vasculitis and thrombosis (Domingo *et al.*, 1992; Lipscomb *et al.*, 1994, 1996; Kennedy *et al.*, 1992; Soto *et al.*, 2011b). The usual pattern of microscopic findings associated with toxoplasmosis in CeMV-infected Mediterranean striped dolphins include granulomatous necrotizing lymphadenitis, adrenalitis, pneumonia and encephalitis. Toxoplasma

cysts can be visualized in affected tissues, and toxoplasma antigen can be demonstrated by immunohistochemistry in inflammatory lesions (Domingo *et al.*, 1992; Soto *et al.*, 2011b). Opportunistic herpesvirus infection in dolphins can cause lymphoid depletion, interstitial pneumonia and non-suppurative encephalitis, with the formation of syncytial cells and nuclear viral inclusions in different tissues (Soto *et al.*, 2012). In harbour seals with herpesvirus infection, hepatic and adrenocortical necrosis, interstitial pneumonia and non-suppurative encephalitis can be observed (Borst *et al.*, 1996; Gulland *et al.*, 1997).

Cases of CeMV-infection restricted to the CNS lack the characteristic microscopic lesions of morbilliviral disease occurring in lungs and lymphoid tissues. Therefore, diagnosis is based on recognition of inflammatory brain lesions, and detection of CeMV-antigen or nucleic acid in the CNS (Domingo *et al.*, 1995; Soto *et al.*, 2011a). Lesions in the brain are often chronic in nature, with perivascular mononuclear inflammatory infiltrates, spongiotic changes in affected cerebral cortex and malacia in some cases. Distribution of lesions and CeMV antigen is variable, being in general most prominent in the cerebral cortex than in the thalamus or brain stem. The cerebellum was usually unaffected. In some cases, CeMV-antigen may be widespread in brain regions in neuronal bodies and processes, showing no or very low inflammatory changes.

17.6 Diagnosis

Diagnosis of morbilliviral disease in marine mammals is based on detection of specific microscopic lesions in target organs (see above), demonstration of morbilliviral antigen and/or viral nucleic acid in tissues and virus isolation (Kennedy *et al.*, 1989; Domingo *et al.*, 1992; Barrett *et al.*, 1993; Nielsen *et al.*, 2008).

Monoclonal antibodies (MoAbs) have been raised by several groups for identification of morbillivirus in cell culture and tissues. For example, MoAb panels against CDV were applied to tissues of infected animals at the beginning of the North Sea seal epizootic, and absence of reaction with some of the individual MoAbs pointed clearly to a new aetiological agent causing the die-off (Örvell *et al.*, 1990; Rima *et al.*, 1992). Similarly, a MoAb panel against PDV was used in dolphin tissues infected with CeMV, showing a different staining pattern than the one in seals infected with PDV. In addition, antibodies, monoclonal as well as polyclonal, have been applied for diagnosis in immunohistochemical techniques to demonstrate morbillivirus antigens in tissues of affected animals (Kennedy *et al.*, 1988a, 1989; Likhoshway *et al.*, 1989; Domingo *et al.*, 1992; Lipscomb *et al.*, 1994). Antibodies against CDV, PDV or MeV may show cross reactivity with other viruses within the genus. For example, PDV has been successfully detected using anti-CDV or anti-MeV antibodies (Kennedy *et al.*, 1988, 1989), and CDV and PDV have been successfully detected using anti-PDV antibodies (Stanton *et al.*, 2004). CeMV has been demonstrated with anti-PDV or anti-CDV antibodies (Domingo *et al.*, 1992; Soto *et al.*, 2011b), but PDV and CDV could not be detected using anti-CeMV (Stanton *et al.*, 2004). In our experience, sensitivity of immunohistochemistry is high enough for routine diagnosis of CeMV field cases. Taking into account the known gradient of mRNA transcription of morbilliviruses, decreasing from the N-protein mRNA to the L-protein mRNA (Ray and Fujinami, 1987), immunohistochemical techniques based on the viral N protein may show advantages over techniques based on other viral proteins that are transcribed later.

Several protocols for molecular diagnosis of morbillivirus infection in marine mammals have been developed. The most frequently used techniques, with targeted gene, amplicon size, viral specificity and reference, are shown in Table 17.2. Most of these techniques use primers designed on conserved regions of the targeted genes. Classic protocols are based on conventional RT-PCR, but quantitative

Table 17.2. RT-PCR techniques for detection of morbilliviruses in marine mammals.

Virus detected	Targeted gene	Technique	Primer sequences (5′–3′) forward (F), reverse (R)	Size	References
PDV	F gene	RT-PCR	F3: GGGGACAGTGCTTCAGCCTATTAAG F4: CAGCCCTAGCTTCTGACCCACGATA	370 bp	Haas *et al.* (1990)
PDV	P gene	RT-PCR	P1: AGAATATCGATGAATCAC P2: CCTATTACTTGGAGGGCC	245 bp	Haas *et al.* (1991)
PDV	H gene	qRT-PCR	F: ACCTCGATGGGCAATGTGTT R: GTCTTACCGTAGATCCCTTCTGAGAT	115 bp	Hammond *et al.* (2005)
CeMV	P gene	RT-PCR	F: ATGTTTATGATCACAGCGGT R: ATTGGGTTGCACCACTTGTC	429 bp	Barrett *et al.* (1993)
CeMV, PDV	P gene	RT-PCR	P1: ATTGGGTTGCACCACTTGTC P2: ATTAAAAAGGG(G/C) ACAGGAGAGAGATCAGCC	78 bp	Krafft *et al.* (1995)
CeMV	N gene	RT-PCR	F: CGGAGACCGAGTCTTCATT R: ATTGGGTTGCACCACTTGTC	230 bp	Taubenberger *et al.* (2000)
	P gene		F: CCHAGRATYGCTGAAATGATHTGTGA R: AACTTGTTCTGRATWGAGTTYTC	378 bp	
CeMV	F gene	qRT-PCR	F: GGCACCATAATTAGCCAGGA R: GCCCAGATTTGTGCCTACAT	192 bp	Bellière *et al.* (2010)
DMV[a]	N gene	RT-PCR, qRT-PCR	F: TGCCAGTACTCCAGGGAACATCCTTC R: TTGGGTCGTCAGTGTTGTCGGACCGTT	173 bp	Grant *et al.* (2009)
PMV[a]			F: GTCTAGTGCTCCGGGGAATATCCCTA R: CTGGATCATCAGCGTTGTCAGATTGCC		
DMV[a], PMV[a]	F gene	qRT-PCR	F: TCTAGAGAATGTCAGGAGAAGTTC R: AAGCTCCTGCACATACAAGGAT	71bp	Rubio-Guerri *et al.* (2013)
PWMV[a]			F: GGAGGGGCAATGTATATAGGG R: CAGCAGTATATCAACAGGCATAGA	74bp	

[a] DMV, PMV and PWMV are CeMV strains.

(qRT-PCR) techniques have also been developed for both PDV and CeMV.

The RT-PCR protocols available for the diagnosis of morbillivirus in seals based on the gene P (see Table 17.2) can amplify sequences from both PDV and CDV (Haas *et al.*, 1991; Krafft *et al.*, 1995). Determining the accurate aetiology for a distemper outbreak in seals is critical for understanding the epidemiology of the outbreak and for implementing management strategies. Sequence analysis of the RT-PCR products can be used to differentiate PDV from CDV (Stanton *et al.*, 2004).

Most of the protocols developed for CeMV do not differentiate between the three recognized strains of the virus (DMV, PMV and PWMV). Grant *et al.* (2009) developed primers on variable regions of the sequence of the N gene being able to differentiate DMV from PMV, but this method doesn't detect PWMV. An UPL-RT-PCR that detects DMV, PMV and PWMV in a single test has been recently described (Rubio-Guerri *et al.*, 2013). It is not known if PWMV would be recognized by this RT-PCR assay.

The available techniques may be successfully used for diagnostic purposes, but the RT-PCR developed by Krafft *et al.* (1995) may be particularly useful for detection of PDV and CeMV in samples showing moderate autolysis, and in partially degraded nucleic acid extracted from paraffin-embedded tissue, due to the small size of the amplicon.

Unusual CNS-restricted forms of morbilliviral disease (Soto *et al.*, 2011a) may need a more complex RT-PCR diagnostic approach. In our experience, 3 of 6 DMV-CNS-localized cases (all of them intensely positive for IHC) were negative when a routine RT-PCR protocol targeting a 429 bp fragment of the P gene (Barrett *et al.*, 1993) was applied. These cases were clearly positive when another

RT-PCR, targeting a much smaller fragment of 78 bp of the same gene (Krafft *et al.*, 1995), was applied (Soto *et al.*, 2011a). The reason for the lack of amplification with the first pair of primers is unknown, but the presence of mutations or other alterations in the virus gene sequence may be responsible.

Exposure of marine mammals to morbilliviruses can be detected by serological techniques. Different techniques have been used in pinnipeds and cetaceans, but results are difficult to interpret and to compare between laboratories, due to the differences in reagents and protocols used (Hall, 1995). Availability of sensitive and specific serological techniques is a crucial step for understanding the epidemiology of phocid and cetaceans morbilliviruses.

The virus neutralization test (VNT) and enzyme-linked immunosorbent assay (ELISA) have been used for this purpose in most studies, and the wide antigenic cross-reactivity existing within morbilliviruses has been exploited to detect antibodies elicited by one specific morbillivirus using related (heterologous) viruses as antigenic substrate (De Vries *et al.*, 1988; Visser *et al.*, 1990, 1993; Barrett *et al.*, 1992). On the other hand, this cross-reactivity negatively affects the specificity of any of these tests, and comparative testing of several morbilliviruses is necessary for the purpose of identifying the virus infecting a given population.

In seals, CDV is commonly used as antigen in indirect ELISA (Osterhaus *et al.*, 1989c; Bostock *et al.*, 1990). A competitive ELISA (cELISA) using CDV as antigen has been developed to detect antibodies against PDV in seals (Saliki and Lehenbauer, 2001), but this test also reacts when cetacean sera containing DMV antibodies are tested. Therefore, ELISA techniques do not allow discrimination between antibodies elicited by morbilliviruses in marine mammals. In spite of that, ELISA techniques using homologous antigens for detecting specific antibodies, either to CeMV or PDV, should be preferred. Such approach has been used in cetaceans by Van Bressem *et al.* (1998), who used purified DMV as antigen in indirect ELISA (iELISA) to detect morbillivirus antibodies in several cetacean species.

Although time-consuming, VNT comparing titres to different morbilliviruses remains the technique of choice to discriminate, as titres are highest against the homologous, infecting virus (Liess *et al.*, 1989). Müller *et al.* (2000) showed that sera from harbour porpoises (*Phocoena phocoena*) from the German Baltic and North Sea showed higher VNT titres against CeMV than against CDV. Also, higher VNT titres to PDV than to CDV have been measured in different seal species in the Atlantic Ocean in North America and Canada (Duignan *et al.*, 1995), showing that PDV is the prevalent morbillivirus in phocids. These and other studies have led to the commonly accepted view that CeMV infects cetaceans and PDV pinnipeds, and that PDV is the usual morbillivirus infecting phocids. However, most studies do not compare titres to heterologous viruses in parallel.

In summary, minimal requirements for morbilliviral disease diagnosis in marine mammals include (i) examination of the carcass and histopathological detection of pneumonia, depletion of lymphocytes in lymphoid organs and encephalitis and (ii) detection of morbilliviral antigen or nucleic acid in affected tissues. CNS should be included in the pathological investigation, to allow the detection of chronic nervous forms occurring in some cetacean species.

References

Aguilar, A. and Borrell, A. (1994) Abnormally high polychlorinated biphenyl levels in striped dolphins (*Stenella coeruleoalba*) affected by the 1990–1992 Mediterranean epizootic. *Science of the Total Environment* 54, 237–247.

Appel, M.J.G., Gibbs, E.P.J., Ter Meulen, S.J.M., Rima, B.K., Stephenson, J.R. and Taylor, W.P. (1981) Morbillivirus disease of animals and man. In: Kustak, E. and Kustak, C. (eds) *Comparative Diagnosis of Viral Diseases*, Vol. 4. Academic Press, New York, pp. 235–297.

Axthelm, M. K. and Krakowka, S. (1998) Experimental old dog encephalitis (ODE) in a gnotobiotic dog. *Veterinary Pathology* 35, 527–534.

Aznar, F.J., Perdiguero, D., Pérez del Olmo, A., Repullés, A., Agustí, C. and Raga, J.A. (2005) Changes in epizoic crustacean infestations during cetacean die-offs: the mass mortality of Mediterranean striped dolphins *Stenella coeruleoalba* revisited. *Diseases of Aquatic Organisms* 67, 239–247.

Baker, J.R. (1992) The pathology of phocine distemper. *Science of the Total Environment* 115, 1–7.

Banyard, A.C., Grant, R.J., Romero, C.H. and Barrett, T. (2008) Sequence of the nucleocapsid gene and genome and antigenome promoters for an isolate of porpoise morbillivirus. *Virus Research* 132, 213–219.

Barrett, T., Crowther, J., Osterhaus, A.D., Subbarao, S.M., Groen, J., Haas, L., Mamaev, L.V., Titenko, A.M., Visser, I.K. and Bostock, C.J. (1992) Molecular and serological studies on the recent seal virus epizootics in Europe and Siberia. *Science of Total Environment* 115, 117–132.

Barrett, T., Visser, I.K.G., Mamaev, L.V., Goatley, L., Van Bressem, M.F. and Osterhaus, A.D.M.E. (1993) Dolphin and porpoise morbilliviruses are genetically distinct from phocine distemper virus. *Virology* 193, 1010–1012.

Barrett, T., Blixenkrone-Møller, M., Di Guardo, G., Domingo, M., Duignan, P., Hall, A., Mamaev, L. and Osterhaus, A.D. (1995) Morbilliviruses in aquatic mammals: report on round table discussion. *Veterinary Microbiology* 44, 261–265.

Beineke, A., Puff, C., Seehusen, F. and Baumgärtner, W. (2009) Pathogenesis and immunopathology of systemic and nervous canine distemper. *Veterinary Immunology and Immunopathology* 127, 1–18.

Beineke, A., Siebert, U., Wohlsein, P. and Baumgärtner, W. (2010) Immunology of whales and dolphins. *Veterinary Immunology and Immunopathology* 133, 81–94.

Bellière, E.N., Esperón, F., Arbelo, M., Muñoz, M.J., Fernández, A. and Sánchez-Vizcaíno, J.M. (2010) Presence of herpesvirus in striped dolphins stranded during the cetacean morbillivirus epizootic along the Mediterranean Spanish coast in 2007. *Archives of Virology* 155, 1307–1311.

Birkun, A., Kuiken, T., Krivokhizhin, S., Haines, D.M., Osterhaus, A.D.M.E., Van de Bildt, M.W.G., Joiris, C.R. and Siebert, U. (1999) Epizootic of morbilliviral disease in common dolphins (*Delphinus delphis ponticus*) from the Black Sea. *Veterinary Record* 144, 85–92.

Blixenkrone-Møller, V., Svansson, P., Have, A., Bötner, J. and Nielsen, J. (1989) Infection studies in mink seal-derived morbillivirus. *Archives of Virology* 106, 165–170.

Blixenkrone-Møller, M., Bolt, G., Gottschalck, E. and Kenter, M. (1994) Comparative analysis of the gene encoding the nucleocapsid protein of dolphin morbillivirus reveals its distant evolutionary relationship to measles virus and ruminant morbilliviruses. *Journal of General Virology* 75, 2829–2834.

Blixenkrone-Møller, M., Bolt, G., Jensen, T.D., Harder, T. and Svansson, V. (1996) Comparative analysis of the attachment protein gene (H) of dolphin morbillivirus. *Virus Research* 40, 47–55.

Bolt, G., Alexandersen, S. and Blixenkrone-Møller, M. (1995) The phosphoprotein gene of a dolphin morbillivirus isolate exhibits genomic variation at the editing site. *Journal of General Virology* 76, 3051–3058.

Borst, G.H., Walvoort, H.C., Reijnders, P.J., Van der Kamp, J.S. and Osterhaus, A.D. (1996) An outbreak of a herpesvirus infection in harbor seals (*Phoca vitulina*). *Journal of Wildlife Diseases* 22, 1–6.

Bostock, C.J., Barrett, T. and Crowther, J.R. (1990) Characterisation of the European seal morbillivirus. *Veterinary Microbiology* 23, 351–360.

Cabezón, O., Resendes, A.R., Domingo, M., Raga, J.A., Agustí, C., Alegre, F., Mons, J.L., Dubey, J.P. and Almería, S. (2004) Seroprevalence of *Toxoplasma gondii* antibodies in wild dolphins from the Spanish Mediterranean coast. *Journal of Parasitology* 90, 643–644.

Caswell, J.L. and Williams, K.J. (2007) Respiratory system. In: Maxie, M.G. (ed.) *Jubb, Kennedy, and Palmer's Pathology of Domestic Animals*, vol. 2. Elsevier Saunders, Edinburgh, UK, pp. 539–699.

Cattaneo, R., Schmid, A., Spielhofer, P., Kaelin, K., Baczko, K., ter Meulen, V., Pardowitz, J., Flanagan, S., Rima, B. K. and Udem, S.A. (1989) Mutated and hypermutated genes of persistent measles viruses which caused lethal human brain diseases. *Virology* 173, 415–425.

Connolly, J.H., Allen, IV., Hurwitz, L.J. and Millar, J.H.D. (1967) Measles virus antibody and antigen in subacute sclerosing panencephalitis. *Lancet* 289, 542–544.

Cosby, S.L., McQuaid, S., Duffy, N., Lyons, C., Rima, B.K., Allan, G.M., McCullough, S.J. and Kennedy, S. (1988) Characterization of seal morbillivirus. *Nature* 336, 115–116.

Cowan, D.F. and Smith, T.L. (1999) Morphology of complex lymphoepithelial organs of the anal canal ('anal tonsil') in the bottlenose dolphin, *Tursiops truncatus*. *Journal of Morphology* 223, 263–268.

Daoust, P.Y., Haines, D.M., Thorsen, J., Duignan, P.J. and Geraci, J.R. (1993) Phocine distemper in a harp seal (*Phoca groenlandica*) from the Gulf of St. Lawrence, Canada. *Journal of Wildlife Diseases* 29, 114–117.

De Swart, R.L., Ross, P.S., Timmerman, H.H., Vos, H.W., Reijnders, P.J., Vos, J.G. and Osterhaus, A.D. (1995) Impaired cellular immune response in harbour seals (*Phoca vitulina*) feeding on environmentally contaminated herring. *Clinical and Experimental Immunology* 101, 480–486.

De Vries, P., UytdeHaag, F.G. and Osterhaus, A.D. (1988) Canine distemper virus (CDV) immune-stimulating complexes (Iscoms), but not measles virus iscoms, protect dogs against CDV infection. *Journal of General Virology* 69, 2071–2083.

Di Guardo, G., Di Francesco, C.E., Eleni, C., Cocumelli, C., Scholl, F., Casalone, C., Peletto, S., Mignone, W., Tittaelli, C., Di Nocera, F., Leonardi, L., Fernández, A., Marcer, F. and Mazzariol, S. (2013) Morbillivirus infection in cetaceans stranded along the Italian coastline: pathological, immunohistochemical and biomolecular findings. *Research in Veterinary Science* 94, 132–137.

Dietz, R., Heide-Jörgensen, M.-P. and Härkönen, T. (1989) Mass deaths of harbor seals (*Phoca vitulina*) in Europe. *Ambio* 18, 258–264.

Domingo, M., Ferrer, L., Pumarola, M., Marco, A., Plana, J., Kennedy, S., McAliskey, M. and Rima, B.K. (1990) Morbillivirus in dolphins. *Nature* 348, 21.

Domingo, M., Visa, J., Pumarola, M., Marco, A., Ferrer, L., Rabanal, R. and Kennedy, S. (1992) Pathologic and immunocytochemical studies of morbillivirus infection in striped dolphins (*Stenella coeruleoalba*). *Veterinary Pathology* 29, 1–10.

Domingo, M., Vilafranca, M., Visa, J., Prats, N., Trudgett, A. and Visser, I. (1995) Evidence for chronic morbillivirus infection in the Mediterranean striped dolphin (*Stenella coeruleoalba*). *Veterinary Microbiology* 44, 229–239.

Duignan, P., Geraci, J.R., Raga, J. and Calzada, N. (1992) Pathology of morbillivirus infection in striped dolphins (*Stenella coeruleoalba*) from Valencia and Murcia, Spain. *Canadian Journal of Veterinary Research* 56, 242–248.

Duignan, P., Sadove, J.S., Saliki, J.T. and Geraci, J.R. (1993) Phocine distemper in harbour seals (*Phoca vitulina*) from Long Island. New York. *Journal of Wildlife Diseases* 29, 465–469.

Duignan, P.J., Saliki, J.T., St Aubin, A.D., Early, G., Sadove, S., House, J.A., Kovacs, K. and Geraci, J.R. (1995) Epizootiology of morbillivirus infection in North American harbor seals (*Phoca vitulina*) and gray seals (*Halichoerus grypus*). *Journal of Wildlife Diseases* 31, 491–501.

Earle, P.J.A., Melia, M.M., Doherty, N.V., Nielsen, O. and Cosby, S.L. (2011) Phocine distemper virus in seals, east coast, United States, 2006. *Emerging Infectious Diseases* 17, 215–220.

Fernández, A., Esperón, F., Herráez, P., Espinosa de los Monteros, A., Clavel, C., Bernabé, A., Sánchez-Vizcaino, J.M., Verborgh, P., Destephanis, R., Toledano, F. and Bayón, A. (2008) Morbillivirus and pilot whale deaths, Mediterranean Sea. *Emerging Infectious Diseases* 14, 792–794.

Forsyth, M.A., Kennedy, S., Wilson, S., Eybatov, T. and Barrett, T. (1998) Canine distemper virus in a Caspian seal. *Veterinary Record* 143, 662–664.

Garg, R.K. (2008) Subacute sclerosing panencephalitis. *Journal of Neurology* 255, 1861–1871.

Gauffier, P. (2008) Estimating the consequences of the 2006–07 morbillivirus epizootic on long-finned pilot whales in the strait of Gibraltar. MSc thesis, Centre National de la Recherche Scientifique–Centre d'Etudes Biologiques de Chizé, Beauvoir-Sur-Niort, France.

Geraci, J.R. (1989) *Clinical Investigations of the 1987–88 Mass Mortality of Bottlenose Dolphins along the U.S. Central and South Atlantic Coast: Final Report to the National Marine Fisheries Service*.

Grachev, M.A., Kumarev, V.P., Mammev, V.P., Zorin, V.L., Baranova, L.V., Denikina, N.N., Belicov, S.I., Petrov, E.A., Kolsnik, V.S., Kolsnik, R.S., Beim, A.M., Kudelin, V.N., Nagieva, F.G. and Sidorovo, V.N. (1989) Distemper virus in Baikal seals. *Nature* 338, 209.

Grant, R.J., Banyard, A.C., Barrett, T., Saliki, J.T. and Romero, C.H. (2009) Real-time RT-PCR assays for the rapid and differential detection of dolphin and porpoise morbilliviruses. *Journal of Virological Methods* 156, 117–123.

Gulland, F.M., Lowenstine, L.J., Lapointe, J.M., Spraker, T. and King, D.P. (1997) Herpesvirus infection in stranded Pacific harbor seals of coastal California. *Journal of Wildlife Diseases* 33, 450–458.

Haas, L., Barrett, T., Harder, T. and Bostock, C.J. (1990) Detection of phocine distemper virus using the polymerase chain reaction. *Deutsche Tierarztliche Wochenschrift* 97, 93–95.

Haas, L., Subbarao, S.M., Harder, T., Liess, B. and Barrett, T. (1991) Detection of phocid distemper virus RNA in seal tissues using slot hybridization and the polymerase chain reaction amplification assay: genetic evidence that the virus is distinct from canine distemper virus. *Journal of General Virology* 72, 825–832.

Hall, A.J. (1995) Morbilliviruses in marine mammals. *Trends in Microbiology* 3, 4–9.

Hammond, J.A., Pomeroy, P.P., Hall, A.J. and Smith, V.J. (2005) Identification and real-time PCR quantification of Phocine distemper virus from two colonies of Scottish grey seals in 2002. *Journal of General Virology* 86, 2563–2567.

Harder, T., Willhaus, T.H., Frey, H.R. and Liess, B. (1990) Morbillivirus infections of seals during the 1988 epidemic in the Bay of Heligoland: III. Transmission studies of cell culture-propagated phocine distemper virus in harbour seals (*Phoca vitulina*) and a grey seal (*Halichoerus grypus*): clinical, virological and serological results. *Journal of Veterinary Medicine Series B* 37, 641–650.

Harder, T.C., Willhaus, T., Leibold, W. and Liess, B. (1992) Investigations on course and outcome of phocine distemper virus infection in harbour seals (*Phoca vitulina*) exposed to polychlorinated biphenyls. Virological and serological investigations. *Zentralblatt für Veterinärmedizin Series B* 39, 19–31.

Harder, T.C., Stede, M., Willhaus, T., Schwarz, J., Heidemann, G. and Liess, B. (1993) Morbillivirus antibodies of maternal origin in harbour seal pups (*Phoca vitulina*). *Veterinary Record* 19, 632–633.

Harwood, J., Carter, S.D., Hughes, D.E., Bell, S.C., Baker, J.R. and Cornwell, H.J.C. (1989) Seal disease predictions. *Nature* 339, 670.

Headley, S.A., Amude, A.M., Alfieri, A.F., Bracarense, A.P.F.R.L., Alfieri, A.A. and Summers, B.A. (2009) Molecular detection of canine distemper and the immunohistochemical characterization of the neurologic lesions in naturally occurring old dog encephalitis. *Journal of Veterinary Diagnostic Investigation* 21, 588–597.

Henderson, G., Trudgett, A., Lyons, C. and Ronald, K. (1992) Demonstration of antibodies in archival sera from Canadian seals reactive with a European isolate of phocine distemper virus. *Science of the Total Environment* 115, 93–98.

Hernández, M., Robinson, I., Aguilar, A., González, L.M., López-Jurado, L.F., Reyero, M.I., Cacho, E., Franco, J., López-Rodas, V. and Costas, E. (1998) Did algal toxins cause monk seal mortality? *Nature* 393, 28–29.

ICTV (2011) ICTVdb index of viruses (online). International Committee on Taxonomy of Viruses, UK (http://ictvonline.org).

Jäger, M., Liess, B., Harder, T., Ising, S. and Stoye, M. (1990) Experimental inoculation of Beagle dogs permits serological differentiation of phocine distemper and canine distemper virus. *Wiener Tierärztliche Monatsschrift* 77, 105–108.

Jauniaux, T., Charlier, G., Desmecht, M., Haelters, J., Jacques, T., Losson, B., Van Gompel, J., Tavernier, J. and Coignoul, F. (2000) Pathological findings in two fin whales (*Balaenoptera physalus*) with evidence of morbillivirus infection. *Journal of Comparative Pathology* 123, 198–201.

Jensen, T., van de Bildt, M., Dietz, H.H., Andersen, T.H., Hammer, A.S., Kuiken, T. and Osterhaus, A. (2002) Another phocine distemper outbreak in Europe. *Science* 297, 209.

Kajiwara, N., Watanabe, M., Wilson, S., Eybatov, T., Mitrofanov, I.V., Aubrey, D.G., Khuraskin, L.S., Miyazaki, N. and Tanabe, S. (2008) Persistent organic pollutants (POPs) in Caspian seals of unusual mortality event during 2000 and 2001. *Environmental Pollutants* 152, 431–442.

Kennedy, S. (1998) Morbillivirus infections in aquatic mammals. *Journal of Comparative Pathology* 119, 201–225.

Kennedy, S., Smyth, J.A., McMullough, S.J., Allan, G.M. and McNeilly, F. (1988a) Confirmation of cause of recent seal deaths. *Nature* 335, 464.

Kennedy, S., Smyth, J.A., Cush, P.F., McCullough, S.J., Allan, G.M. and McQuaid, S. (1988b) Viral distemper now found in porpoises. *Nature* 336, 21.

Kennedy, S., Smyth, J.A., Cush, P.F., Duignan, P., Plateen, M., McMullough, S.J. and Allan, G. (1989) Histopathologic and immunocytochemical studies of distemper in Seals. *Veterinary Pathology* 26, 97–103.

Kennedy, S., Smyth, J.A., Cush, P.F., McAliskey M., McCullough, S.J. and Rima, B.K. (1991) Histological and immunocytochemical studies of distemper in harbour porpoises. *Veterinary Pathology* 28, 1–7.

Kennedy, S., Kuiken, T., Ross, H.M., McAliskey, M., Moffett, D., McNiven, M. and Carole, M. (1992) Morbillivirus infection in two common porpoises (*Phocoena phocoena*) from the coasts of England and Scotland. *Veterinary Record* 131, 286–290.

Kennedy, S., Kuiken, T., Jepson, P.D., Deaville, R., Forsyth, M., Barrett, T., Van de Bildt, M.W., Osterhaus, A.D., Eybatov, T., Duck, C., Kydyrmanov, A., Mitrofanov, I. and Wilson, S. (2000) Mass die-off of Caspian seals caused by canine distemper virus. *Emerging Infectious Diseases* 6, 637–639.

Krafft, A., Lichy, J.H., Lipscomb, T.P., Klaunberg, B.A., Kennedy, S. and Taubenberger, J.K. (1995) Postmortem diagnosis of morbillivirus infection in bottlenose dolphins (*Tursiops truncatus*) in the Atlantic and Gulf of Mexico epizootics by polymerase chain reaction-based assay. *Journal of Wildlife Diseases* 31, 410–415.

Kuiken, T., Kennedy, S., Barrett, T., Van de Bildt, M.W., Borgsteede, F.H., Brew, S.D., Codd, G.A., Duck, C., Deaville, R., Eybatov, T., Forsyth, M.A., Foster, G., Jepson, P.D., Kydyrmanov, A., Mitrofanov, I., Ward, C.J.,

Wilson, S. and Osterhaus, A.D. (2006) The 2000 canine distemper epidemic in Caspian seals (*Phoca caspica*): pathology and analysis of contributory factors. *Veterinary Pathology* 43, 321–338.

Liess, B., Frey, H.R., Zaghawa, A. and Stede, M. (1989) Morbillivirus infection of seals (*Phoca vitulina*) during the 1988 epidemic in the Bay of Heligoland. I. Mode, frequency and significance of cultural virus isolation and neutralizing antibody detection. *Zentralblatt für Veterinärmedizin. Series B* 36, 601–608.

Likhoshway, Ye.V., Grachev, M.A., Kumarev, V.P., Solodun, Yu.V., Goldberg, O.A., Belykh, O.I., Nagieva, F.G., Nikulina, V.G. and Kolesnik, B.S. (1989) Baikal seal virus. *Nature* 339, 266.

Lipscomb, T.P., Schulman, F.Y., Moffett, D. and Kennedy, S. (1994) Morbilliviral disease in Atlantic bottlenose dolphins (*Tursiops truncatus*) from the 1987–1988 epizootic. *Journal of Wildlife Diseases* 30, 567–571.

Lipscomb, T.P., Kennedy, S., Moffett, D., Krafft, A., Klaunberg, B.A., Lichy, J.H., Regan, G.T., Worthy, G.A.J. and Taubenberger, J.K. (1996) Morbilliviral epizootic in bottlenose dolphins of the Gulf of Mexico. *Journal of Veterinary Diagnostic Investigation* 8, 283–290.

Lipscomb, T.P., Mense, M.G., Habecker, P.L., Taubenberger, J.K. and Schoelkopf, R. (2001) Morbilliviral dermatitis in seals. *Veterinary Pathology* 38, 724–726.

Lyons, C., Welsh, M.J., Thorsen, J., Ronald, K. and Rima, B.K. (1993) Canine distemper virus isolated from a captive seal. *Veterinary Record* 132, 487–488.

Mamaev, L.V., Visser, I.K., Belikov, S.I., Denikina, N.N., Harder, T., Goatley, L., Rima, B., Edginton, B., Osterhaus, A.D. and Barrett, T. (1996) Canine distemper virus in Lake Baikal seals (*Phoca sibirica*). *Veterinary Record* 138, 18.

Markussen, N.H. and Have, P. (1992) Phocine distemper virus infection in harp seals (*Phoca groenlandica*). *Marine Mammal Science* 8, 19–26.

Mazzariol, S., Marcer, F., Mignone, W., Serracca L., Goria, M. and Marsili, L. (2012) Dolphin morbillivirus and *Toxoplasma gondii* coinfection in a Mediterranean fin whale (*Balaenoptera physalus*). *BMC Veterinary Research* 8, 20.

Mazzariol, S., Peletto, S., Mondin, A., Centelleghe, C., Di Guardo, G., Di Francesco, C.E., Casalone, C. and Acutis, P.L. (2013) Dolphin morbillivirus infection in a captive harbour seal (*phoca vitulina*). *Journal of Clinical Microbiology* 51, 708–711.

McCullough, S.J., McNeilly, F., Allan, G.M., Kennedy, S., Smyth, J.A., Cosby, S.L., McQuaid, S. and Rima, B.K. (1991) Isolation and characterisation of a porpoise morbillivirus. *Archives of Virology* 118, 247–252.

Müller, G., Siebert, U., Wünschmann, A., Artelt, A. and Baumgärtner, W. (2000) Immunohistological and serological investigation of morbillivirus infection in harbour porpoises (*Phocoena phocoena*) from the German Baltic and North Sea. *Veterinary Microbiology* 75, 17–25.

Müller, G., Wohlsein, P., Beineke, A., Haas, L., Greiser-Wilke, I., Siebert, U., Fonfara, S., Harder, T., Stede, M., Gruber, A.D. and Baumgärtner, W. (2004) Phocine distemper in German seals, 2002. *Emerging Infectious Diseases* 10, 723–725.

Müller, G., Kaim, U., Haas, L., Greiser-Wilke, I., Wohlsein, P., Siebert, U. and Baumgärtner, W. (2008) Phocine distemper virus: characterization of the morbillivirus causing the seal epizootic in northwestern Europe in 2002. *Archives of Virology* 153, 951–956.

Nielsen, O., Smith, G., Weingartl, H., Lair, S. and Measures, L. (2008) Use of a SLAM transfected vero cell line to isolate and characterize marine mammal morbilliviruses using an experimental ferret model. *Journal of Wildlife Diseases* 44, 600–611.

Olsson, M., Karlsson, B. and Ahnland, E. (1994) Diseases and environmental contaminants in seals from the Baltic and the Swedish west coast. *Science of the Total Environment* 154, 217–227.

Örvell, C., Blixenkrone-Møller, M., Svansson, V. and Have, P. (1990) Immunological relationships between phocid and canine distemper virus studied with monoclonal antibodies. *Journal of General Virology* 71, 2085–2092.

O'Shea, T.J. (2000) Cause of seal die-off in 1988 is still under debate. *Science* 290, 1097.

Osterhaus, A.D.M.E. and Vedder, E.J. (1988) Identification of virus causing recent seal deaths. *Nature* 335, 20.

Osterhaus, A.D.M.E., Groen, J., UytdeHaag, F.G.C.M., Visser, I.K.G., Van de Bildt, M.W.G., Bergman, A. and Kligeborn, B. (1989a) Distemper virus in Baikal seals. *Nature* 338, 209–210.

Osterhaus, A.D.M.E., Broeders, H.W.J., Groen, J., UytdeHaag, F.G.C.M., Visser, I.K.G., van de Bilt, M.W.G., Örvell, C., Kumarev, V.P. and Zorin, V.L. (1989b) Different morbilliviruses in European and Siberian seals. *Veterinary Record* 125, 647–648.

Osterhaus, A.D., Groen, J., UytdeHaag, F.G., Visser, I.K., Vedder, E.J., Crowther, J. and Bostock, C.J. (1989c) Morbillivirus infections in European seals before 1988. *Veterinary Record* 125, 326.

Osterhaus, A.D.M.E., De Swart, R.L., Vos, H.W., Ross, P.S., Kenter, M.J.H. and Barrett, T. (1995) Morbillivirus infections of aquatic mammals: newly identified members of the genus. *Veterinary Microbiology* 44, 219–227.

Osterhaus, A., Groen, J., Niesters, H., Van de Bildt, M., Martina, B., Vedder, L., Vos, J., Egmond, H., Sidi, B.A. and Barhan, M.E.O. (1997) Morbillivirus in monk seal mass mortality. *Nature* 388, 838–839.

Osterhaus, A., van de Bildt, M., Vedder, L., Martina, B., Niesters, H., Vos, J., van Egmond, H., Liem, D., Baumann, R., Androukaki, E., Kotomatas, S., Komnenou, A., Abou Sidi, B., Jiddou, A.B. and Barham, M.E. (1998) Monk seal mortality: virus or toxin? *Vaccine* 16, 979–981.

Philippa, J.D., van de Bildt, M.W., Kuiken, T., 't Hart, P. and Osterhaus, A.D. (2009) Neurological signs in juvenile harbour seals (*Phoca vitulina*) with fatal phocine distemper. *Veterinary Record* 164, 327–331.

Piza, J. (1991) Striped dolphin mortality in the Mediterranean. In: Pastor, X. and Simmonds, M. (eds) *Proceedings of the Mediterranean Striped Dolphin Mortality International Workshop, X*. Greenpeace Mediterranean Sea Project, Palma de Mallorca, Spain, pp. 93–103.

Pohlmeyer, G., Pohlenz, J. and Wohlsein, P. (1993) Intestinal lesions in experimental phocine distemper: light microscopy, immunohistochemistry and electron microscopy. *Journal of Comparative Pathology* 109, 57–69.

Raga, J.A., Banyard, A., Domingo, M., Corteyn, M., Van Bressem, M.F., Fernández, M., Aznar, F.J. and Barrett, T. (2008) Dolphin morbillivirus epizootic resurgence, Mediterranean Sea. *Emerging Infectious Diseases* 14, 471–473.

Ray, J. and Fujinami, R.S. (1987) Characterization of in vitro transcription and transcriptional products of measles virus. *Journal of Virology* 61, 3381–3387.

Rijks, J.M., Read, F.L., van de Bildt, M.W., van Bolhuis, H.G., Martina, B.E., Wagenaar, J.A., van der Meulen, K., Osterhaus, A.D. and Kuiken, T. (2008) Quantitative analysis of the 2002 phocine distemper epidemic in the Netherlands. *Veterinary Pathology* 45, 516–530.

Rima, B.K., Curran, M.D. and Kennedy, S. (1992) Phocine distemper virus, the agent responsible for the 1988 mass mortality of seals. *Science of the Total Environment* 115, 45–55.

Rima, B.K., Collin, A.M.J. and Earle, J.A.P. (2005) Completion of the sequence of a cetacean morbillivirus and comparative analysis of the complete genome sequences of four morbilliviruses. *Virus Genes* 30, 113–119.

Ross, P.S., de Swart, R.L., Visser, I.K., Vedder, L.J., Murk, W., Bowen, W.D. and Osterhaus, A.D. (1994) Relative immunocompetence of the newborn harbour seal, *Phoca vitulina*. *Veterinary Immunology and Immunopathology* 42, 331–348.

Rubio-Guerri, C., Melero, M., Rivera-Arroyo, B., Belliére, E.N., Crespo, J.L., Garciá-Párragd, D., Esperón F. and Sánchez-Vizcaino, J.M. (2013) Simultaneous diagnosis of cetacean morbillivirus infection in dolphins standard in the Spanish Mediterranean sea in 2011 using a novel Universal Probe Library (UPL) RT-PCR array. Veterinary Microbiology 165, 109–114.

Saliki, J.T. and Lehenbauer, T.W. (2001) Monoclonal antibody-based competitive enzyme-linked immunosorbent assay for detection of morbillivirus antibody in marine mammal sera. *Journal of Clinical Microbiology* 39, 1877–1881.

Schulman, F.Y., Lipscomb, T.P., Moffett, D., Krafft, A.E., Lichy, J.H., Tsai, M.M., Taubenberger, J.K. and Kennedy, S. (1997) Histologic, immunohistochemical, and polymerase chain reaction studies of bottlenose dolphins from the 1987–1988 United States Atlantic coast epizootic. *Veterinary Pathology* 34, 288–295.

Soto, S., Alba, A., Ganges, L., Vidal, E., Raga, J.A., Alegre, F., González, B., Medina, P., Zorrilla, I., Martínez, J., Marco, A., Pérez, M., Pérez, B., Pérez de Vargas Mesas, A., Martínez Valverde, R. and Domingo, M. (2011a) Post-epizootic chronic dolphin morbillivirus infection in Mediterranean striped dolphins (*Stenella coeruleoalba*). *Diseases of Aquatic Organisms* 96, 187–194.

Soto, S., González, R., Alegre, F., González, B., Medina, P., Raga, J.A., Marco, A. and Domingo, M. (2011b) Epizootic of dolphin morbillivirus on the Catalonian Mediterranean coast in 2007, *Veterinary Record* 169, 101.

Soto, S., González, B., Willoughby, K., Maley, M., Olvera, A., Kennedy, S., Marco, A. and Domingo, M (2012) Systemic herpesvirus and morbillivirus co-infection in a striped dolphin (*Stenella coeruleoalba*). *Journal of Comparative Pathology* 146, 269–273.

Stanton, J.B., Brown, C.C., Poet, S., Lipscomb, T.P., Saliki, J. and Frasca, S. Jr (2004) Retrospective differentiation of canine distemper virus and phocine distemper virus in phocids. *Journal of Wildlife Diseases* 40, 53–59.

Stone, B.M., Blyde, D.J., Saliki, J.T., Blas-Machado, U., Bingham, J., Hyatt, A., Wang, J., Payned, J. and Cramerid, S. (2011) Fatal cetacean morbillivirus infection in an Australian offshore bottlenose dolphin (*Tursiops truncatus*). *Australian Veterinary Journal* 89, 452–457.

Tapparel, C., Maurice, D. and Roux, L. (1998) The activity of Sendai virus genomic and antigenomic promoters requires a second element past the leader template regions: a motif (GNNNNN)3 is essential for replication. *Journal of Virology* 72, 3117–3128.

Taubenberger, J.K., Tsai, M., Krafft, A.E., Lichy, J.H., Reid, A.H., Schulman, F.Y. and Lipscomb, T.P. (1996) Two morbilliviruses implicated in bottlenose dolphin epizootics. *Emerging Infectious Diseases* 2, 213–216.

Taubenberger, J.K., Tsai, M.M., Atkin, T.J., Fanning, T.G., Krafft, A.E., Moeller, R.B., Kodsi, S.E., Mense, M.G. and Lipscomb, T.P. (2000) Molecular genetic evidence of a novel morbillivirus in a long-finned pilot whale (*Globicephala melas*). *Emerging Infectious Diseases* 1, 42–45.

Trudgett, A., Lyons, C., Welsh, M.J., Duffy, N., McCullough, S.J. and McNeilly, F. (1991) Analysis of a seal and a porpoise morbillivirus using monoclonal antibodies. *Veterinary Record* 128, 61.

Van Bressem, M.F., Van Waerebeek, K., Fleming, M. and Barrett, T. (1998) Serological evidence of morbillivirus infection in small cetaceans from the Southeast Pacific. *Veterinary Microbiology* 59, 89–98.

Van Bressem, M.F., Van Waerebeek, K. and Raga, J.A. (1999) A review of virus infections of cetaceans and the potential impact of morbilliviruses, poxviruses and papillomaviruses on host population dynamics. *Diseases of Aquatic Organisms* 38, 53–65.

Van Bressem, M.F., Van Waerebeek, K., Jepson, P.D., Raga, J.A., Duignan, P.J., Nielsen, O., Di Beneditto, A.P., Siciliano, S., Ramos, R., Kant, W., Peddemors, V., Kinoshita, R., Ross, P.S., Lopez-Fernández, A., Evans, K., Crespo, E. and Barrett, T. (2001) An insight into the epidemiology of dolphin morbillivirus worldwide. *Veterinary Microbiology* 81, 287–304.

Van Bressem, M.F., Raga, J.A., Di Guardo, G., Jepson, P.D., Duignan, P.J., Siebert, U., Barrett, T., Santos, M.C., Moreno, I.B., Siciliano, S., Aguilar, A. and Van Waerebeek, K. (2009) Emerging infectious diseases in cetaceans worldwide and the possible role of environmental stressors. *Diseases of Aquatic Organisms* 86, 143–157.

Van de Bildt, M.W., Martina, B.E., Vedder, E.J., Androukaki, E., Kotomatas, S., Komnenou, A., Sidi, B.A., Jiddou, A.B., Barham, M.E., Niesters, H.G. and Osterhaus, A.D. (2000) Identification of morbilliviruses of probable cetacean origin in carcases of Mediterranean monk seals (*Monachus monachus*). *Veterinary Record*, 146, 691–694.

Van de Bildt, M.W., Martina, B.E., Sidi, B.A. and Osterhaus, A.D. (2001) Morbillivirus infection in a bottlenosed dolphin and a Mediterranean monk seal from the Atlantic coast of West Africa. *Veterinary Record* 148, 210–211.

Van de Bildt, M.W., Kuiken, T. and Osterhaus, A.D. (2005) Cetacean morbilliviruses are phylogenetically divergent. *Archives of Virology* 150, 577–583.

Visser, I.K., Van Bressem, M.F., de Swart, R.L., Van de Bildt, M.W., Vos, H.W., Van der Heijden, R.W., Saliki, J.T., Orvell, C., Kitching, P., Kuiken, T., Barrett, T. and Osterhaus, A.D.M.E. (1993) Characterization of morbilliviruses isolated from dolphins and porpoises in Europe. *Journal of General Virology* 74, 631–641.

Visser, I.K.G., Kumarev, V.P., Örvell, C., De Vries, P., Broeders, H.W.J., Van de Bildt, M.W.G., Groen, J., Teppema, J.S., Burger, M.C., UytdeHaag, F.G.C.M. and Osterhaus, A.D.M.E. (1990) Comparison of two morbilliviruses isolated from seals during outbreaks of distemper in North West Europe and Siberia. *Archives of Virology* 111, 149–164.

Wong, T.C., Ayata, M., Hirano, A., Yoshikawa, Y., Tsuruoka, H. and Yamanouchi, K. (1989) Generalized and localized biased hypermutation affecting the matrix gene of a measles virus strain that causes subacute sclerosing panencephalitis. *Journal of Virology* 63, 5464–5468.

Yang, W.-C, Pang, V.F, Jeng, C.R., Chou, L.-S. and Chueh, L.-L. (2006) Morbilliviral infection in a pygmy sperm whale (*Kogia breviceps*) from Taiwanese waters. *Veterinary Microbiology* 116, 69–76.

18 Sendai Virus

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18.1 Historical Perspective

The discovery of Sendai virus dates back to the spring of 1952, during an outbreak of fatal pneumonitis in newborn children at Tohoku University Hospital, in the city of Sendai, Japan. To isolate the causative organism, Kuroya and colleagues (1953) intra-nasally inoculated mice with tissue homogenates from lungs of infants who underwent autopsy. All inoculated mice succumbed to infection within 1 week and had consolidated lung lesions similar to those seen with influenza infection. Homogenates prepared from infected mouse lungs were then used to inoculate embryonated chicken eggs, after which the amniotic fluid obtained showed haemagglutinating activity with chicken erythrocytes. Haemagglutination inhibition testing performed with known haemagglutinating viruses, such as mumps, Newcastle virus and influenza showed that none of these viruses could interfere with the haemagglutinating activity of this new isolate. Thus, the new isolate was termed 'Newborn virus pneumonitis (type Sendai)' (Kuroya *et al.*, 1953; Sano *et al.*, 1953). Between 1952 and 1955, multiple publications suggested an association between human influenza-like illness and the isolation of this novel virus by egg or mouse inoculation.

In 1954, Fukumi and colleagues published a separate report describing a new pneumotropic virus isolated from mouse-passaged influenza virus strains. This novel virus exhibited haemagglutinating activity similar to influenza virus, but had different antigenic components, as determined by haemagglutination inhibition, cross neutralization tests in developing eggs and complement fixation reactions (Fukumi *et al.*, 1954). Also, this virus was similar to the strains they had received from laboratories throughout Japan, which were thought to have been isolated from human patients with a febrile respiratory illness. However, on further investigation, it was observed that laboratory mice were used for virus isolation in each instance. Fukumi *et al.* (1954) suggested that, due to nationwide spread of this pneumotropic, haemagglutinating virus in laboratory mice in 1953, it was feasible that this agent, later named Sendai virus, was not the actual cause of the 1952 human neonatal pneumonitis outbreak, but rather a separate murine virus altogether. The true aetiologic agent of the fatal pneumonia outbreak in newborns remains unknown (Fukumi *et al.*, 1954).

One year later, at the third annual Japan Society for Virology meeting, the 'Newborn virus pneumonitis (type Sendai)' was given the formal designation, 'Haemagglutinating

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virus of Japan (JHV)'. However, the International Committee on Taxonomy of Virus later formally adopted the name 'Sendai virus'. Several publications later described the antigenic similarities between Sendai virus and human parainfluenza virus type 1. In 1958, the Virus Subcommittee of the International Nomenclature Committee approved the proposal for naming the parainfluenza viruses, including Sendai virus, as part of the parainfluenza type 1 virus group. Sendai virus has since become synonymous with murine parainfluenza type 1 (Andrews *et al.*, 1959; Cook and Chanock, 1963; Lyn *et al.*, 1991; Nagai *et al.*, 2011).

18.2 Epidemiology

Sendai virus infects a broad range of animals, with severe disease and mortality described clearly only in mice (Parker *et al.*, 1978). In laboratory mouse colonies, Sendai virus associated disease is seen in epizootic and enzootic patterns (Ishida and Homma, 1978; Faisca and Desmecht, 2007; Nagai *et al.*, 2011). The epizootic pattern, or clinically apparent infection, occurs when Sendai virus is introduced into a colony for the first time. The virus appears suddenly and spreads rapidly, resulting in acute clinical infection with a high mortality rate in nearly all susceptible rodents. Symptoms in mice include ruffled coat, hunched appearance, crust formation around the eyes, teeth chattering and death. The virus then disappears from the colony over a period of a few months, transitioning to enzootic infections over time in breeding colonies (Bhatt and Jonas, 1974; Zurcher *et al.*, 1977; Ishida and Homma, 1978; Baker, 1998; Faisca and Desmecht, 2007).

The enzootic pattern, or clinically silent infection, typically occurs shortly after weaning when maternal antibodies are waning. Mice show few clinical symptoms, however the majority of susceptible animals will be infected. After recovery, virus persists in the infected colony, resulting in perpetuation of infection as new, susceptible animals are added (either commercially or via breeding). Cessation of breeding will ultimately result in elimination of infection if no additional animals are added to the colony (Profeta *et al.*, 1969; Ishida and Homma, 1978; Faisca and Desmecht, 2007).

Sendai virus infections in laboratory mice, both epizootic and enzootic, have been reported in the USA, UK, Japan, Canada and France (Parker *et al.*, 1964; Parker and Reynolds, 1968; Descoteaux *et al.*, 1977; Carthew and Verstraete, 1978; Zenner and Regnault, 2000; Gannon and Carthew, 1980). Infections have also been documented in laboratory rats, guinea pigs and hamsters. Identification of Sendai virus infection in pigs illustrates that the tropism is not limited to rodent hosts (Profeta *et al.*, 1969; Ishida and Homma, 1978; Baker, 1998; Zenner and Regnault, 2000; Park *et al.*, 2006; Easterbrook *et al.*, 2008; Pritchett-Corning *et al.*, 2009).

The first outbreak of swine Sendai infection was documented in 1953 near Tokyo, Japan in pigs presenting with an influenza-like illness. By 1955, nearly half of the tested pigs in a slaughterhouse in Tokyo had serum antibodies to Sendai virus. One year later, there were seropositive pigs noted in 15 different regions of Japan, a situation with the potential to result in an agricultural disaster. Interestingly, since 1960, there have been no additional seropositive pigs reported in Japan (Ishida and Homma, 1978; Nagai *et al.*, 2011).

18.3 Transmission and Susceptibility

In susceptible rodents, Sendai virus infection is highly contagious; direct contact with an infected rodent and/or contaminated fomite is the main route of transmission (Parker and Reynolds, 1968). Transmission studies have shown that when uninfected mice are caged with infected mice, all susceptible animals are infected within 1 week. All uninfected mice in cages adjoining infected mice, where nasal-oral contact occurred, were also infected a few days later, supporting direct contact as route of transmission (Parker and Reynolds, 1968).

Several factors influence susceptibility of mice to Sendai virus infection, including age and mouse strain. Mice born to dams immune to Sendai virus receive maternal antibody primarily through suckling, with documented protection from infection during these first few weeks. This protection, however, is short-lived and by 3–6 weeks of age, the mice are again susceptible to Sendai infection (Iida *et al.*, 1973; Bhatt and Jonas, 1974).

Genetic susceptibility also varies by laboratory mouse strain. The strains of mice most commonly studied in Sendai infection include 129/J, DBA/2, C57BL/6, C3HeN, SJL and Balb/c (Parker *et al.*, 1978; Brownstein *et al.*, 1981; Faisca *et al.*, 2005). Table 18.1 compares and contrasts the susceptibility of these strains to Sendai virus infection. Of these strains, 129/J mice are the most susceptible to severe Sendai infection, and exhibit clinical signs of pneumonia, tachypnoea, mortality, heavier lung weights and more weight loss when compared to the other strains when challenged with identical virus inoculum. An acute inflammatory response can be seen by day 6–7 of infection, with foci of macrophage infiltration of the alveoli, thickened peribronchial and perivascular cuffing, and diffused epithelial hyperplasia, consistent with a histopathologic diagnosis of severe bronchial epithelial hyperplasia and diffuse interstitial pneumonia (Parker *et al.*, 1978; Faisca *et al.*, 2005).

DBA/2 mice, when infected with Sendai virus, also show clinical signs of pneumonia, with weight loss, tachypnoea and mortality. On histopathologic examination, there is hyperplasia and desquamation of the epithelial lining, alveolar and luminal exudate consisting of cell debris, epithelial cells, lymphoid cells, neutrophils and macrophages, cellular infiltrate of the lamina propria consistent with a diagnosis of severe, purulent, necrotizing bronchiolitis with multi-focal alveolitis (Parker *et al.*, 1978; Brownstein *et al.*, 1981; Faisca *et al.*, 2005; Suryadevara *et al.*, 2011) (Fig. 18.1). Similar histopathologic evaluation can be seen in C3H/HeN, C57BL/6 and SJL mice; however, these mice exhibit a much less severe clinical infection. Infected C57BL/6 mice have less weight loss and less mortality when compared with the previously mentioned 129/J and DBA/2 mice. C3H/HeN and SJL mice actually maintain their weights during infection (Parker *et al.*, 1978; Brownstein *et al.*, 1981; Faisca *et al.*, 2005).

Balb/C mice have a benign, asymptomatic course with Sendai virus infection. There is little, if any, weight loss, and repressed viral replication with rapid virus clearance. On histopathologic examination, the airways resemble healthy

Table 18.1. Comparison of clinical signs of illness and histopathologic changes in the lungs of different strains of mice infected with Sendai virus. (Parker *et al.*, 1978; Brownstein *et al.*, 1981; Faisca *et al.*, 2005.)

Mouse strain	Severity of clinical infection	Clinical signs of illness	Histopathologic pulmonary changes
Balb/c	Benign	Asymptomatic	Slight increase in interstitial cell density, few foci of mononuclear infiltration of the lamina propria
C3HeN SJL	Mild	Asymptomatic	Epithelial hyperplasia, desquamation, luminal and alveolar exudate, lymphoid infiltration of the lamina propria
C57BL/6		Weight loss	
DBA/2	Severe	Tachypnoea, weight loss, death	
129/J			Macrophage infiltration of alveoli, thick peribronchial/perivascular cuffing, epithelial hyperplasia

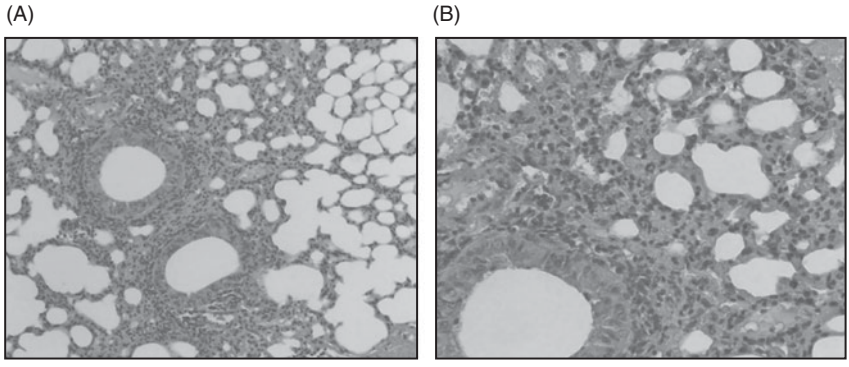

Fig. 18.1. Histologic examination of lungs from DBA/2 mice day 5 post-inoculation with Sendai virus. (A) 20× and (B) 40× images showing increased peribronchiolar neutrophil and lymphocyte infiltration.

murine lungs without luminal or alveolar exudates and with intact epithelium. There may be a slight increase in interstitial cell density and few foci of mononuclear infiltration in the lamina propria (Parker *et al.*, 1978; Brownstein *et al.*, 1981; Faisca *et al.*, 2005).

18.4 Virology

18.4.1 Virus structure

Sendai virus is a non-segmented, single-stranded, negative-sense RNA virus, which belongs to the *Respirovirus* genus of the *Paramyxoviridae* family. The virus particle consists of an outer lipid bilayer membrane surrounding a helical nucleocapsid (Fig. 18.2). Protruding from the outer membrane are two glycoproteins, haemagglutinin-neuraminidase (HN) and fusion (F). The matrix (M) protein crosslinks and stabilizes the outer glycoproteins and the inner helical nucleocapsid. The major components of the nucleocapsid include the virus genome encapsidated by the nucleocapsid protein (NP) and the RNA polymerase complex, consisting of the large (L) protein and the phosphoprotein (P) (Tashiro and Homma, 1983; Fouillot-Coriou and Roux, 2000; Chanock *et al.*, 2001; Faisca and Desmecht, 2007; Nagai *et al.*, 2011).

18.4.2 Genome

The Sendai virus genome, consisting of 15,384 nucleotides, starts with a short 3′ leader region adjacent to the genes encoding the six structural proteins in the order NP, P, M, F, HN, L, followed lastly by a short 5′ trailer region (Table 18.2, Fig. 18.3). Five of these genes are monocistronic, as they have one open reading frame (ORF), one start codon and translate a single protein product. The P gene, however, is polycistronic with overlapping ORFs and RNA editing, and expresses multiple proteins (P, V, C, C′, Y1, Y2). The P gene ORF spans most of the gene, and results in translation of the P protein. The V ORF is nearly identical to the P ORF; however, with RNA editing, the addition of a guanosine residue results in an mRNA that expresses the cysteine-rich V protein. The C ORF has four separate start codons and expresses C, C′, Y1, and Y2 proteins, collectively referred to as the C protein. Of these proteins expressed by the P gene, the P protein is the most critical to genome replication, the V protein inhibits viral RNA synthesis and the C proteins interact with host-cell STAT1 to interfere with interferon signaling, thus evading innate antiviral responses and slowing viral clearance (Vidal *et al.*, 1990; Curran *et al.*, 1991; Kato *et al.*, 1997, 2004, 2007; Strahle *et al.*, 2003; Kiyotani *et al.*, 2007).

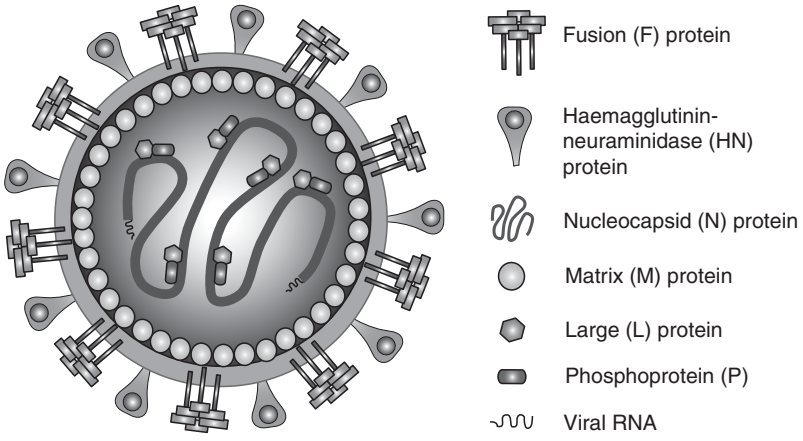

Fig. 18.2. Schematic representation of Sendai virus particle, consisting of the outer lipid bilayer membrane, the HN and F proteins protruding from the membrane, and the M protein cross-linking the HN and F proteins with the inner nucleocapsid.

Table 18.2. Sendai virus strain 52 gene products and functions, GenBank AY909550.1, NCBI Reference Sequence NC_001552.1.

Coding regions	Length (base pairs)	Length (amino acids)	Gene product(s)	Primary function(s)
NP	1575	524	Nucleoprotein	Involved in RNA synthesis
P	1707	568	Phosphoprotein (P)	Involved in RNA synthesis
	1154		V protein	Inhibits RNA synthesis
	615, 648, 546, 528		C proteins (C, C′, Y1, Y2)	Reduce antiviral effects of interferon
	955		W protein	Unclear at this time
M	1047	348	Matrix protein	Cross-links outer glycoproteins and inner nucleocapsid Important in viral assembly and budding Outer envelope formation
F	1698	565	Fusion glycoprotein	Fusion of virion to host cell membrane Outer envelope formation
HN	1728	575	Haemagglutinin-neuraminidase glycoprotein	Adsorption of virion to host cell membrane Cleavage of terminal sialic acid for release of progeny virion from cell surface Outer envelope formation
L	6687	2228	L protein	Involved in RNA synthesis

18.4.3 Virus entry

Sendai virus infection is initiated by the binding of the HN glycoprotein to the sialo-oligosaccharide-containing receptor of glycopeptides or gangliosides on the host-cell surface. HN, a homo-tetramer composed of disulfide-linked dimers, is a trans-membrane protein with an N-terminal cyto-plasmic tail (stalk region) and a C-terminal

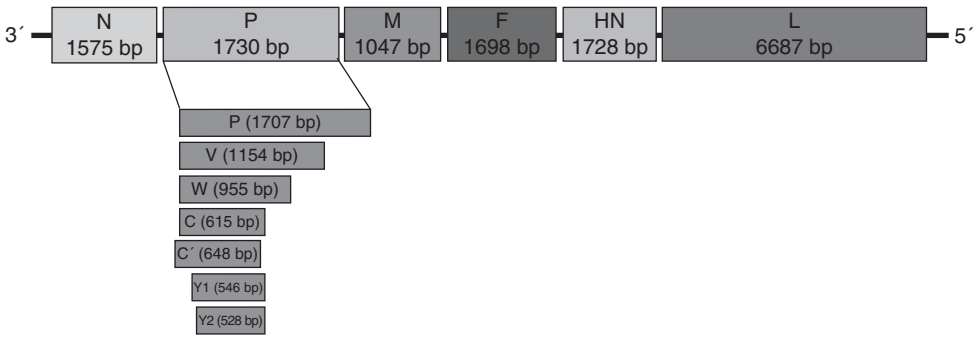

Fig. 18.3. Schematic representation of Sendai virus genome. Consisting of approximately 15,384 nucleotides, the genome starts with a 3′-leader region, followed by genes encoding the six structural proteins, followed by a 5′-trailer region.

extracellular globular head domain, which contains the sites for both the binding to the sialic acid receptor and the eventual cleavage of the sialic acid by the neuraminidase component (Moscona, 2005).

The binding of HN to the host-cell receptor not only allows for attachment of the virion to the host cell but is also required to induce the conformational change in the F protein from the pre-fusion state to the post-fusion state for fusion to occur. The trimeric F protein is a trans-membrane protein, oriented opposite to HN, with an extracellular N-terminal domain and a C-terminal membrane domain and cytoplasmic tail. Initially synthesized as the inactive precursor F_0, post-translational cleavage between Arg116 and Phe117 into F_1 and F_2 subunits by cellular proteases is required for biologic activity. Fusion of the virion to the cell membrane, which occurs at neutral pH, releases the viral genome into the cytoplasm (Tashiro and Homma, 1983; Tashiro *et al.*, 1990; Chanock *et al.*, 2001; Moscona, 2005; Lamb *et al.*, 2006).

18.4.4 Transcription

Upon entering the cytoplasm, the viral RNA remains encapsidated by the NP protein. The viral RNA-dependent RNA polymerase binds to the NP-RNA to form a complex consisting of 30 molecules of the L protein, 300 molecules of the P protein and 2600 molecules of the NP protein, now ready for transcription. Initiation of transcription occurs with the entrance of the RNA-polymerase at the 3′ terminus of the genome, transcribing first positive strand RNA (55 nucleotides), followed by the six genes (NP, P, M, F, HN and L) whose mRNA are then modified by capping and methylation at the 5′ terminus and polyadenylation at the 3′ terminus (Ogino *et al.*, 2005; Faisca and Desmecht, 2007; Murphy and Grdzelishvili, 2008).

The P and L proteins, together forming the RNA polymerase complex, are critical to genomic transcription. The P protein mediates binding of the polymerase to the NP protein, while the L protein is capable of the enzymatic activities required for both RNA transcription and processing, including initiation, elongation, termination of transcription and replication, as well as capping, cap methylation and polyadenylation (Bowman *et al.*, 1999; Ogino *et al.*, 2005).

18.4.5 Assembly and budding

Virus assembly and budding, processes mediated by the M protein, take place in a series of steps. The progeny genome, NP protein, and L and P proteins of the RNA polymerase complex combine to form the helical nucleocapsid. The HN and F glycoproteins are transported to and randomly inserted into the host-cell plasma membrane.

The accumulation of the M protein in the cytoplasm results in re-positioning of the viral glycoproteins around the location of the M protein. This modified area of the plasma membrane is now a recognition site for the NP protein. The M protein then cross-links the glycoproteins and the nucleocapsid. Budding occurs as the host-cell membrane forms the outer viral envelope surrounding the nucleocapsid. Lastly, the neuraminidase component of the HN glycoprotein removes the terminal sialic acid from the HN and F glycoproteins, allowing for release of the progeny virus from the cell surface (Ali and Nayak, 2000; Fouillot-Coriou and Roux, 2000; Chanock *et al.*, 2001; Nagai *et al.*, 2011).

18.4.6 Virus strains

There are multiple strains of Sendai virus used in laboratory experiments, including but not limited to MN, Z, KN, Mol, Hm, DDN, GGP, SEE-19, M-73, P, Hamamatsu, Ohita, Strain 52, Enders and Cantell strains. There is some antigenic variation among the strains, as described by Yamaguchi *et al.* (1989), who studied 13 of the strains isolated between 1953 and 1983. Unlike the strains isolated in 1953–4, the strains isolated after 1976 lacked reactivity with HN- or F-specific antibodies (Yamaguchi *et al.*, 1989). Yamaguchi showed that following experimental intra-nasal inoculation of mice with various Sendai virus strains, the MN, KN and Mol strains resulted in mortality and lung consolidation, while mice infected with Z and Hm all survived, with lung consolidation seen only in mice inoculated with high doses of the Z strain, suggesting a difference in virulence among strains (Yamaguchi *et al.*, 1988). Pathogenic strains of Sendai virus are rapidly attenuated during tissue culture passage such that mouse passaged isolates are used in research infection models.

18.5 Pathogenesis

Sendai virus specifically targets the epithelial mucosa of the respiratory tract, including the nose, trachea, bronchi and bronchioles, with minimal involvement of subepithelial tissue. Many organ systems have Sendai virus-specific receptors; however, only the respiratory tract becomes infected, as there is no viraemia. The pneumotropism of this virus is thought to be due to the presence of host proteases in the lung, which cleave the F protein into its active form and therefore produce an infectious virion (Tashiro and Homma, 1983; Tashiro *et al.*, 1990).

Following experimental intra-nasal inoculation of mice, Sendai virus antigens can be detected on the luminal surface of bronchial-mucosal cells on day 1, and in the cytoplasm of these cells by day 2. The number and intensity of viral antigen staining of affected cells increases over time. Peak viral antigen detection in the lungs occurs between days 3 and 6, with no detectable viral antigen by day 10. Of note, no viral antigen is detected deep to the basement membrane, providing evidence of superficial epithelial involvement (Blandford and Heath, 1972; Charlton and Blandford, 1977).

Peak lung virus titres are detected between days 3 and 5 after inoculation (Blandford and Heath, 1972; Castleman *et al.*, 1987; Faisca and Desmecht, 2007; Simon *et al.*, 2011). Virus titres then decrease rapidly and are no longer detected in the lungs by day 10–14 (Castleman *et al.*, 1987; Simon *et al.*, 2011).

Using light and electron microscopy of infected rat lungs, leukocyte infiltration, including neutrophils, macrophages and lymphocytes, into pulmonary airways can be detected as early as day 2 after inoculation, with peak inflammation between days 4 and 8 (Castleman *et al.*, 1987; Simon *et al.*, 2011). Viral nucleocapsids and budding virions in ciliated and non-ciliated bronchiolar epithelial cells, with accumulation of cytoplasmic nucleocapsids resulting in hypertrophy of these cells, can be seen on day 3 (Castleman, 1984). By day 5, bronchiolar epithelial hyperplasia with epithelial necrosis, sloughing and leukocyte infiltration can result in luminal obstruction (Castleman, 1984). Epithelial repair and resolution of pulmonary inflammation can

be seen by day 14–17. Sendai virus is a potent inducer of gamma-interferon (IFN-γ), interleukin-2 (IL-2), tumour necrosis factor (TNF), IL-6 and IL-10, peak concentrations of which coincide with viral clearance from the lungs (Castleman *et al.*, 1987; Mo *et al.*, 1995; Simon *et al.*, 2011).

The adaptive host defence to infection with Sendai virus involves both humoral and cellular responses. Antibody-expressing cells appear early in infection in the perivascular and peribronchial areas of the lung. By day 2 of infection, there is an increase in IgM, IgG and IgA seen in the pulmonary infiltrates, with a predominance of IgA until day 5. Between days 5 and 27, there are equal proportions of IgA and IgG, whereas after day 27, there again is predominance of IgA (Blandford and Heath, 1974).

Virus-specific antibody production in the cervical and mediastinal lymph nodes can be seen after the first week of infection, with peak IgM at day 7 followed by peak IgG at day 10, after which there is a decrease in both antibody classes. Antibody detection in the spleen is further delayed; peak titres of all three classes were observed between 14 and 24 days post-infection (Charlton and Blandford, 1977; Sangster *et al.*, 1995).

Serologic evidence of Sendai infection is seen by day 7, with peak IgM and IgG by day 10, after which IgM titres decrease and disappear by day 27, whereas IgG titres remain elevated. Unlike the antibody production in pulmonary infiltrates, serum IgA titres remain low throughout infection (Sangster *et al.*, 1995).

T cells also play a critical role in the immune response to Sendai virus infection, both in their direct involvement in virus clearance and in their stimulation of other immune mediators. Significant increase in cytotoxic activity in the spleen can be detected by day 4 post-infection, with a peak at day 6 to 7. Increased cytotoxic activity over background activity persists for 3–5 weeks after infection (Anderson *et al.*, 1977, 1979).

Simon *et al.* (2011) showed that mice resistant to severe Sendai infection, such as C57BL/6, produced a more pronounced T-cell response, including influx of $CD4^+$, $CD8^+$ and NK cells, without a strong IFN response, for effective viral clearance. On the other hand, mice susceptible to severe Sendai infection, such as DBA/2, induced less T-cell response with a more robust interferon response, resulting in more severe lung disease (Simon *et al.*, 2011; Suryadevara *et al.*, 2012). Additional markers of Sendai virus severity remain to be explored.

18.6 Diagnosis

Serologic testing, including enzyme-linked immunosorbent assay (ELISA), immunofluorescence, haemagglutination (HA), haemagglutination-inhibition (HI) and complement fixation (CF), is primarily performed for the diagnosis of Sendai virus (Parker and Reynolds, 1968; Profeta *et al.*, 1969; Bhatt and Jonas, 1974; Park *et al.*, 2006; Becker *et al.*, 2007; Easterbrook *et al.*, 2008; Pritchett-Corning *et al.*, 2009).

ELISAs are the most commonly used serologic assays because of the high sensitivity to detection of early antibody, with positive results as early as 7 days post-infection. ELISAs are easy to use, standardized and less costly when compared to the other serologic testing methods (Parker *et al.*, 1979; Rottinghaus *et al.*, 1986; Suzuki *et al.*, 1987; Compton and Riley, 2001). Immunofluorescence, with its high sensitivity, rapidity and low cost, is an available alternative to ELISAs. A disadvantage of this method is the lack of standardization, as interpretation of the results is highly subjective and observer dependent (Compton and Riley, 2001). HA, HI and CF tests are less sensitive, less specific and less standardized, with day-to-day variability, when compared to ELISAs and immunofluorescence (Parker *et al.*, 1979; Faisca and Desmecht, 2007).

Sendai virus can also be isolated in cell culture, typically BHK-21 cells, primary monkey kidney cells (PMK) or VERO cells, with confirmation of virus presence by determination of cytopathic effect, haemadsorption of guinea pig red blood cells, immunofluorescence or immunohistochemcial staining. More recently, the development of reverse-transcriptase polymerase chain reaction (RT-PCR) has allowed

for rapid testing and confirmation of infection in symptomatic animals, with quantification of virus titre by real-time PCR (Bhatt and Jonas, 1974; Parker *et al.*, 1978; MacLahan and Dubovi, 2011).

18.7 Control of Infection

When a colony of rodents becomes infected with Sendai virus, several methods have been shown to be effective in controlling infection. Sacrificing infected animals, cessation of breeding until the virus is eliminated, disinfection of the cages and serologic surveillance of the colony will ultimately encourage eradication of the virus. All animals being introduced into the newly cleansed colony should be screened prior to placement or re-breeding with appropriate quarantine measures employed for all new members (Saito *et al.*, 1980; Eaton *et al.*, 1982; MacLahan and Dubovi, 2011).

18.8 Sendai Virus Infection to Model Human PIV Illness

Human parainfluenza viruses (hPIV), the aetiology for a third of the acute lower respiratory tract infections in children, cause a spectrum of illness from mild upper respiratory infection and otitis media to severe laryngotracheobronchitis and bronchiolitis. hPIV can be detected in up to 30% of children hospitalized for acute respiratory tract infection, second in aetiology of infection only to respiratory syncytial virus (RSV) (Henrickson, 2003). There are currently no vaccines or anti-viral medications proven to be effective in the prevention or treatment of hPIV infection, although early phase I clinical trials in infants show some promise for a live, attenuated PIV type 3 vaccine.

Sendai virus and hPIV 1, both members of the parainfluenza virus 1 group, produce clinically significant respiratory disease in rodents and humans, respectively. Experimental animal models have used Sendai virus infection to replicate and describe hPIV pathogenesis. Similar to children with hPIV infection who develop acute airway inflammation, tachypnoea and wheezing, mice infected with Sendai virus exhibit clinical signs of respiratory infection with weight loss, tachypnoea and increased airway hyperresponsiveness, as measured by whole body plethysmography (Suryadevara *et al.*, 2011).

The respiratory epithelium is the site of binding and infection of both hPIV and Sendai virus, as directed by the HN protein. Subsequent infection is secondary to the combination of direct virus destruction and the resulting immune response. Local cytokine production, as detected from nasal wash specimens of children infected with hPIV, includes interleukin-6 (IL-6), IL-1β, CCL3 (MIP-1α), CCL4 (MIP-1β), CXCL9, MIG and CCL5 (RANTES) (El Feghaly *et al.*, 2010). Similarly, studies of Sendai infection in mice have shown increased production of IL-6, IL-1β, MIP-1α, MIG and RANTES, in addition to IFN-γ, TNF-α, IL-2 and IL-10 (Mo *et al.*, 1995; Simon *et al.*, 2011; Suryadevara *et al.*, 2012), thus showing the consistency of clinical presentation and inflammatory response to species-specific parainfluenza infection in their natural hosts and the usefulness of animal models.

Respiratory virus infections, including both hPIV and RSV, in children have been associated with the subsequent development of asthma, although the exact role of these viruses in chronic lung disease is still unclear (Stein *et al.*, 1999; Gern, 2004; Carroll and Hartert, 2008). While there are mouse models currently being used to study the pathobiology of viral infections, asthma and even RSV-induced wheezing, there is little data regarding the pathogenesis of the effect of hPIV infection on chronic lung diseases, such as asthma. The use of Sendai infection in the mouse, as a model for hPIV infection in children, can be expanded to address both the acute infectious process as well as the chronic pulmonary changes that occur with infection and that can have a critical impact on the lives of both children and adults.

References

Ali, A. and Nayak, D.P. (2000) Assembly of Sendai virus: M protein interacts with F and HN proteins and with the cytoplasmic tail and transmembrane domain of F protein. *Virology* 276, 289–303.

Anderson, M.J., Bainbridge, D.R., Pattison, J.R. and Heath, R.B. (1977) Cell-mediated immunity to Sendai virus infection in mice. *Infection and Immunity* 15, 239–244.

Anderson, M.J., Pattison, J.R. and Heath, R.B. (1979) The nature of the effector cells of cell-mediated immune responses to Sendai and Kunz virus infection in mice. *British Journal of Experimental Pathology* 60, 314–319.

Andrews, C.H., Bang, F.B., Chanock, R.M. and Zudanov, V.M. (1959) Parainfluenza viruses 1, 2, and 3: suggested names for recently described myxoviruses. *Virology* 8, 129–130.

Baker, D.G. (1998) Natural pathogens of laboratory mice, rats, and rabbits and their effects on research. *Clinical Microbiology Reviews* 11, 234–265.

Becker, S.D., Bennett, M., Stewart, J.P. and Hurst, J.L. (2007) Serological survey of virus infection among wild house mice (*Mus domesticus)* in the UK. *Laboratory Animals* 41, 229–238.

Bhatt, P.N. and Jonas, A.M. (1974) An epizootic of Sendai infection with mortality in a barrier-maintained mouse colony. *American Journal of Epidemiology* 100, 222–229.

Blandford, G. and Heath, R.B. (1972) Studies on the immune response and pathogenesis of Sendai virus infection of mice. I. The fate of viral antigens. *Immunology* 22, 637–649.

Blandford, G. and Heath, R.B. (1974) Studies on the immune response and pathogenesis of Sendai virus infection of mice. II. The immunoglobulin class of plasma cells in the bronchial sub-mucosa. *Immunology* 26, 667–671.

Bowman, M.C., Smallwood, S. and Moyer, A.A. (1999) Dissection of individual functions of the Sendai virus phosphoprotein in transcription. *Journal of Virology* 73, 6474–6483.

Brownstein, D.G., Smith, A.L. and Johnson, E.A. (1981) Sendai virus infection in genetically resistant and susceptible mice. *American Association of Pathologists* 105, 156–163.

Carroll, K.C. and Hartert, T.V. (2008) The impact of respiratory viral infection on wheezing illnesses and asthma exacerbations. *Immunology and Allergy Clinics of North America* 28, 539–561.

Carthew, P. and Verstraete, A. (1978) A serological survey of accredited breeding colonies in the United Kingdom for common rodent viruses. *Laboratory Animals* 12, 245–246.

Castleman, W.L. (1984) Alterations in pulmonary ultrastructure and morphometric parameters induced by parainfluenza (Sendai) virus in rats during postnatal growth. *American Journal of Pathology* 114, 322–335.

Castleman, W.L., Brundage-Anguish, L.F., Kreitzer, L. and Neuenschwander, S.B. (1987) Pathogenesis of bronchiolitis and pneumonia induced in neonatal and weanling rats by parainfluenza (Sendai) virus. *American Journal of Pathology* 129, 277–286.

Chanock, R.M., Murphy, B.R. and Collins, P.L. (2001) Parainfluenza viruses. In: Knipe, D.M. and Howley, P.M. (eds) *Fields Virology*, 4th edn. Lippincott Williams & Wilkins, Philadelphia, Pennsylvania, pp. 1341–1379.

Charlton, D. and Blandford, G. (1977) Immunoglobulin class-specific antibody response in serum, spleen, lungs, and bronchoalveolar washings after primary and secondary virus infection of germfree mice. *Infection and Immunity* 17, 521–527.

Compton, S.R. and Riley, L.K. (2001) Detection of infectious agents in laboratory rodents: traditional and molecular techniques. *Comparative Medicine* 51, 113–119.

Cook, M.K. and Chanock, R.M. (1963) In vivo antigenic studies of parainfluenza viruses. *American Journal of Hygiene* 77, 150–159.

Curran, J., Boeck, R. and Kolakofsky, D. (1991) The Sendai virus P gene expresses both an essential protein and an inhibitor of RNA synthesis of shuffling modules via mRNA editing. *EMBO Journal* 10, 3079–3085.

Descoteaux, J.P., Grignon-Archambault, D. and Lussier, G. (1977) Serologic study on the prevalence of murine viruses in five Canadian mouse colonies. *Laboratory Animal Science* 27, 621–626.

Easterbrook, J.D., Kaplan, J.B., Glass, G.E., Watson, J. and Klein, S.L. (2008) A survey of rodent-borne pathogens carried by wild-caught Norway rats: a potential threat to laboratory rodent colonies. *Laboratory Animals* 42, 92–98.

Eaton, G.J., Lerro, A., Custer, R.P. and Crane, A.R. (1982) Eradication of Sendai pneumonitis from a conventional mouse colony. *Laboratory Animal Science* 32, 384–386.

El Feghaly, R., McGann, L., Bonville, C.A., Branigan, P.J., Suryadevara, M., Rosenberg, H.F. and Domachowske, J.B. (2010) Local production of inflammatory mediators during childhood parainfluenza virus infection. *Pediatric Infectious Disease Journal* 29, e26–e31.

Faisca, P. and Desmecht, D. (2007) Sendai virus, the mouse parainfluenza type 1: a longstanding pathogen that remains up-to-date. *Research in Veterinary Science* 82, 115–125.

Faisca, P., Anh, D.B.T. and Desmecht, D.J.M. (2005) Sendai virus-induced alterations in lung structure/function correlate with viral loads and reveal a wide resistance/susceptibility spectrum among mouse strains. *American Journal of Physiology – Lung, Cell Molecular Physiology* 289, L777–787.

Fouillot-Coriou, N. and Roux, L. (2000) Structure-function analysis of the Sendai virus F and HN cytoplasmic domain: different role for the two proteins in the production of virus particle. *Virology* 270, 464–475.

Fukumi, H., Nishikawa, F. and Kitayama, T. (1954) A pneumotropic virus from mice causing hemagglutination. *Japanese Journal of Medical Science and Biology* 7, 345–363.

Gannon, J. and Carthew, P. (1980) Prevalence of indigenous viruses in laboratory animal colonies in the United Kingdom 1978–1979. *Laboratory Animals* 14, 309–311.

Gern, J.E. (2004) Viral respiratory infection and the link to asthma. *Pediatric Infectious Disease Journal* 23, S78–86.

Henrickson, K.J. (2003) Parainfluenza viruses. *Clinical Microbiology Reviews* 16, 242–264.

Iida, T., Tajima, M. and Murata, Y. (1973) Transmission of maternal antibodies to Sendai virus in mice and its significance in enzootic infection. *Journal of General Virology* 18, 247–254.

Ishida, N. and Homma, M. (1978) Sendai virus. *Advances in Virus Research* 23, 349–383.

Kato, A., Kiyotani, K., Sakai, Y., Yoshida, T. and Nagai, Y. (1997) The paramyxovirus, Sendai virus, V protein encodes a luxury function required for viral pathogenesis. *EMBO Journal* 16, 578–587.

Kato, A., Cortese-Grogan, C., Moyer, S.A., Sugahara, F., Sakaguchi, T., Kubota, T., Otsuki, N., Kohase, M., Tashiro, M., and Nagai, Y. (2004) Characterization of the amino acid residues of Sendai virus C protein that are critically involved in its interferon antagonism and RNA synthesis down-regulation. *Journal of Virology* 78, 7443–7454.

Kato, A., Kiyotani, K., Kubota, T., Yoshida, T., Tashiro, M. and Nagai, Y. (2007) Importance of anti-interferon capacity of Sendai virus C protein for pathogenicity in mice. *Journal of Virology* 81, 3264–3271.

Kiyotani, K., Sakaguchi, T., Kato, A., Nagai, Y. and Yoshida, T. (2007) Paramyxovirus Sendai virus V protein counteracts innate virus clearance through IRF-3 activation but not interferon, in mice. *Journal of Virology* 359, 92–91.

Kuroya, M., Ishida, N. and Shiratori, T. (1953) Newborn virus pneumonitis (type Sendai) II. The isolation of a new virus. *Tohoku Journal of Experimental Medicine* 58, 62.

Lamb, R.A., Paterson, R.G. and Jardetzky, T.S. (2006) Paramyxovirus membrane fusion: lessons from the F and HN atomic structures. *Virology* 344, 30–37.

Lyn, D., Gill, D.S., Scroggs, R.A. and Portner, A. (1991) The nucleoproteins of human parainfluenza virus type 1 and Sendai virus share amino acid sequences and antigenic and structural determinants. *Journal of General Virology* 72, 983–987.

MacLahan, N.J. and Dubovi, E.J. (2011) Paramyxoviridae. In: MacLahan, N.J. and Dubovi, E.J. (eds) *Fenner's Veterinary Virology*. Elsevier, London, pp. 309–310.

Mo, X.Y., Sarawar, S.R. and Doherty, P.C. (1995) Induction of cytokines in mice with parainfluenza pneumonia. *Journal of Virology* 69, 1288–1291.

Moscona, A. (2005) Entry of parainfluenza virus into cells as a target for interrupting childhood respiratory disease. *Journal of Clinical Investigation* 115, 1688–1698.

Murphy, A.M. and Grdzelishvili, V.Z. (2008) Identification of Sendai virus L protein amino acid residues affecting viral mRNA cap methylation. *Journal of Virology* 83, 1669–1681.

Nagai, Y., Takakura, A., Irie, T., Yonemitsu, Y. and Gotoh, B. (2011) Sendai virus: evolution from mouse pathogen to a state-of-the art tool in virus research and biotechnology. In: Samal, S.K. (ed.) *The Biology of Paramyxoviruses*. Caister Academic Press, Norfolk, UK, pp. 115–172.

Ogino, T., Kobayashi, M., Iwama, M. and Mizumoto, K. (2005) Sendai virus RNA-dependent RNA polymerase L protein catalyzes cap methylation of virus-specific mRNA. *Journal of Biological Chemistry* 280, 4429–4435.

Park, J.H., Seok, S.H., Baek, M.W., Lee, H.Y., Kim, D.J., Cho, J.S., Kim, C.K., Hwang, D.Y. and Park, J.H. (2006) Microbiological monitoring of guinea pigs reared conventionally at two breeding facilities in Korea. *Experimental Animals* 55, 427–432.

Parker, J.C. and Reynolds, R.K. (1968) Natural history of Sendai virus infection in mice. *American Journal of Epidemiology* 88, 112–125.

Parker, J.C., Tennant, R.W. and Ward, T.G. (1964) Enzootic Sendai virus infections in mouse breeder colonies within the United States. *Science* 146, 936–938.

Parker, J.C., Whiteman, M.D. and Richter, C.B. (1978) Susceptibility of inbred and outbred mouse strains to Sendai virus and prevalence of infection in laboratory rodents. *Infection and Immunity* 19, 123–130.

Parker, J.C., O'Beirne, A.J. and Collins, M.J. (1979) Sensitivity of enzyme-linked immunosorbent assay, complement fixation, and hemagglutination inhibition serological tests for detection of Sendai virus antibody in laboratory mice. *Journal of Clinical Microbiology* 9, 444–447.

Pritchett-Corning, K.R., Cosentino, J. and Clifford, C.B. (2009) Contemporary prevalence of infectious agents in laboratory mice and rats. *Laboratory Animals* 43, 165–173.

Profeta, M.L., Lief, F.S. and Plotkin, S.A. (1969) Enzootic Sendai infection in laboratory hamsters. *American Journal of Epidemiology* 89, 316–324.

Rottinghaus, A.A., Gibson, S.V. and Wagner, J.E. (1986) Comparison of serological tests for detection of antibodies to Sendai virus in rats. *Laboratory Animal Science* 36, 496–498.

Saito, M., Suzuki, E., Nakayama, K., Matsubara, J. and Nakagawa, M. (1980) An attempt for eradication of Sendai virus from a mouse breeding colony. *Experimental Animals* 29, 457–460.

Sangster, M., Hyland, L., Sealy, R. and Coleclough, C. (1995) Distinctive kinetics of the antibody forming cell response to Sendai virus infection of mice of different anatomical compartments. *Virology* 207, 287–291.

Sano, T., Niitsu, I. and Nakagawa, I. (1953) Newborn virus pneumonitis (Type Sendai) I. The clinical observation. *Tohoku Journal of Experimental Medicine* 58, 56.

Simon, A.Y., Sasaki, N., Ichii, O., Kajino, K., Kon, Y. and Agui, T. (2011) Distinctive and critical roles for cellular immunity and immune-inflammatory response in the immunopathology of Sendai virus infection in mice. *Microbes and Infection* 13, 783–797.

Stein, R.T., Sherrill, D., Morgan, W.J., Holberg, C.J., Halonen, M., Taussig, L.M., Wright, A.L. and Martinez, F.D. (1999) Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. *Lancet* 354, 541–545.

Strahle, L., Garcin, D., Le Mercier, P., Schlaak, J.F. and Kolakofsky, D. (2003) Sendai virus targets inflammatory responses, as well as the interferon-induced antiviral state, in a multifaceted manner. *Journal of Virology* 77, 7903–7913.

Suryadevara, M., Bonville, C.A. and Domachowske, J.B. (2011) Parainfluenza virus causes clinically severe lung infection in mice. Presented at Infectious Disease Society of America, Boston, MA.

Suryadevara, M., Bonville, C.A., Rosenberg, H.F. and Domachowske, J.B. (2012) Parainfluenza infection induces differing inflammatory responses in 2 strains of mice. Submitted to *RSV 2012*.

Suzuki, E., Noguchi, A. and Takeda, H. (1987) Detection of antibodies to Sendai virus in mouse sera by enzyme-linked immunosorbent assay (ELISA). *Japanese Journal of Medical Science and Biology* 40, 109–117.

Tashiro, M. and Homma, M. (1983) Pneumotropism of Sendai virus in relation to protease-mediated activation in mouse lungs. *Infection and Immunity* 39, 879–888.

Tashiro, M., Yamakawa, M., Tobita, K., Klenk, H.D., Rott, R. and Seto, J.T. (1990) Organ tropism of Sendai virus in mice: proteolytic activation of the fusion glycoprotein in mouse organs and budding site at the bronchial epithelium. *Journal of Virology* 64, 3627–3634.

Vidal, S., Curran, J. and Kolakofsky, D. (1990) Editing of the Sendai virus P/C mRNA by G insertion occurs during mRNA synthesis via a virus-encoded activity. *Journal of Virology* 64, 239–246.

Yamaguchi, R., Iwai, H. and Ueda, K. (1988) Variations of virulence and other properties among Sendai virus strains. *Microbiology and Immunology* 32, 235–240.

Yamaguchi, R., Iwai, H. and Ueda, K. (1989) Antigenic variation among Sendai virus strains detected by monoclonal antibodies. *Microbiology and Immunology* 33, 133–139.

Zenner, L. and Regnault, J.P. (2000) Ten-year long monitoring of laboratory mouse and rat colonies in French facilities: a retrospective study. *Laboratory Animals* 34, 76–83.

Zurcher, C., Burek, J.D., Van Nunen, M.C. and Meihuizen, S.P. (1977) A naturally occurring epizootic caused by Sendai virus in breeding and aging rodent colonies. I. Infection in the mouse. *Laboratory Animal Science* 27, 955–962.

19 Pneumonia Virus of Mice

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19.1 Historical Perspective

Pneumonia virus of mice (PVM) is a member of the *Mononegavirales* order, family *Paramyxoviridae*, subfamily *pneumovirinae*, genus *pneumovirus*. The 2009 International Committee on Taxonomy of Viruses includes two other agents in this genus, human and bovine respiratory syncytial viruses (hRSV and bRSV). Given the identification of related pneumoviruses from rodents, cows and humans, it is not surprising that related pneumoviruses have also been isolated from the respiratory tract of other mammalian species. Descriptions of caprine (Lehmkuhl *et al.*, 1980), ovine (Eleraky *et al.*, 2003), cameline (Intisar *et al.*, 2010), and canine (Renshaw *et al.*, 2010) pneumoviruses all appear to indicate that pneumoviruses cause respiratory infections, with little or no indication for spread beyond the respiratory tract.

PVM was the first of the pneumoviruses to be discovered and described. In 1939, Horsfall and Hahn (1939, 1940) described mice with a transmissible form of pneumonitis. Infection was easily passed from sick to healthy mice using lung suspensions as the inoculum. Recipient mice developed pneumonitis following this inoculum, fulfilling Kochs 3rd and 4th postulates for an infectious agent. The agent was dubbed 'pneumonia virus of mice', and more than three decades later, PVM was identified as the first isolated member to be placed in the genus 'pneumovirus'.

19.2 Epidemiology

Since its discovery, PVM has been reported to infect a broad range of rodents, although data from wild rodents are sparse, so little is known regarding the natural history of infection outside of the laboratory. Results from a small number of field studies, however, indicate that nearly half of wild mice, and approximately one third of wild voles, have been infected with PVM (Kaplan *et al.*, 1980). Following discovery of the virus, seroprevalence studies performed on a broad range of laboratory animals indicated that the virus was widespread in research laboratories. In those early studies, more than 85% of laboratory mice, 65% of guinea pigs, 45% of hamsters and 20% of cotton rats were determined to be serum antibody positive. These observations support the hypothesis that PVM is likely to be ubiquitous in the wild, and that natural infection with the virus confers some degree of immune protection during later exposure. Between the mid-1960s and the early 1980s, studies confirmed that PVM infection was extremely common in laboratory rodents, where symptoms of infection

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were most often mild (Parker *et al.*, 1966; Gannon and Carthew, 1980). PVM is no longer an endemic pathogen in most rodent research facilities; its decline can be attributed to the introduction of laminar flow hoods, the use of quarantine periods when introducing new rodent or rodent colonies, the use of pathogen-free rodent distributors together with vigilant sentinel surveillance. In laboratories that study rodent virus pathogens, the introduction of micro-isolation cages containing independent HEPA filtration and adherence to strict infection control procedures greatly limits the potential for inadvertent introduction of PVM into other areas of the facility.

Early studies on the seroprevalence of PVM suggested that some non-rodent species develop detectable antibody responses against PVM, including laboratory rabbits and nonhuman primates (Horsfall and Curnen 1946; Kagiyama *et al.*, 1986). The early observation that laboratory primates had approximately 25% seroprevalence for specific anti-PVM antibody raised the possibility that PVM might also infect humans. Early indications suggested that humans did indeed have detectable anti-PVM antibody (Horsfall and Curnen, 1946), but it was the work by Pringle and Eglin (1986) that argued that the majority of human adults (75%) were seropositive. More recently, Brock *et al.* (2012) demonstrated that the anti-PVM antibody was instead a naturally occurring polyreactive IgG, not at all specific to pneumoviruses. Consistent with their findings, no clinical illness has ever been described in response to a PVM exposure in humans, including none among researchers and animal handlers who routinely handle experimentally infected animals under research protocols.

19.3 Transmission and Susceptibility

19.3.1 Virus strains

Care in describing PVM strains used in laboratory studies has become important since outcomes of studies clearly depend on the strain of virus used. Newly cultured and amplified wild strains of PVM should be similarly described, and comparison of experimental results to historical publications should be undertaken with caution. The prototype virus, PVM strain 15, as archived at the American Type Culture Collection (ATCC) was the first virus to be sequenced. The strain was maintained in tissue culture for nearly 20 years (referred to as PVM strain 15, Warwick) and when used to challenge laboratory mice was found to replicate efficiently but cause little or no clinical illness (Cook *et al.*, 1998). Subsequently, another group obtained PVM strain 15 directly from ATCC (PVM strain 15, ATCC) and showed that it caused significant clinical illness (Krempl and Collins, 2004). A third isolate, designated PVM strain J3666, which has been maintained only through mouse passage, is highly virulent (Thorpe and Easton, 2005).

Little is known regarding PVM transmission in the wild, although infection is very common given the findings of the limited number of serosurveillence studies on wild rodents. In the controlled laboratory setting, PVM infection is established via direct inoculation of the respiratory tract, usually by intranasal inoculation under light anaesthesia. In the original studies by Horsfall and Hahn, lung homogenates from the sixth mouse passage were still lethal to 25% of the recipient mice. Inbred mouse strains differ in their susceptibility to PVM infection, with C57BL/6 and SJL mice being more resistant, and DBA/2 and 129/SV more susceptible (Anh *et al.*, 2006).

19.4 Virology

19.4.1 Virion structure

Evidence that PVM was a pneumovirus was first suggested by electron microscopy (Compans *et al.*, 1967; Berthiaume *et al.*, 1974). The enveloped virion is pleiomorphic, with shapes varying from filamentous to spherical, findings that are also typical of the other members of the pneumovirus genus. Unlike hRSV, which is predominantly

spherical in morphology, the most typical morphology for PVM virions are the filamentous structures of 100–120 nm in diameter with lengths as long as 3 microns. The significance, if any, of this observation is unclear, and may simply reflect the type of host-cell infected (Berthiaume *et al.*, 1974). PVM is a non-segmented, single-stranded, negative-sense RNA virus of the *Paramyxoviridae* family. The virus particle consists of an outer lipid bilayer membrane surrounding a helical nucleocapsid. Three proteins are associated with the vial envelope. The F (fusion) protein expands the lipid membrane with its amino terminus located outside of the virion. The G (attachment) protein, by contrast is a type 2 membrane protein with its carboxy-terminus located outside of the virion. Together, the F and G proteins comprise the 10–14 nm spikes that are observed on the virion surface (Fig. 19.1). The small hydrophic protein (SH) is embedded in the membrane. The capsid contains a single copy of the negative-sense non-segmented genomic RNA that is tightly associated with structural proteins to form the helical nucleocapsid structure. The proteins include the nucleocapsid protein (N), the phosphoprotein (P) and the RNA polymerase (L). This nucleocapsid structure is surrounded by the matrix (M) protein, forming a link between the nucleocapsid and the viral envelope. The carboxy-terminus of the F protein and the amino terminus of the G protein interact with the M protein on the internal surface of the lipid envelope.

19.4.2 The PVM genome

All pneumoviruses have an RNA genome of approximately 15,000 nucleotides (Easton *et al.*, 2004). The complete nucleotide sequences of three PVM strains are available (Krempl *et al.*, 2005; Thorpe and Easton 2005). The negative-sense single-strand RNA genome contains 10 coding regions, NS1, NS2, N, P, M, SH, G, F, M2 and L (Fig. 19.2). Each gene is flanked by a conserved start (AGGAyAArT) and end sequence (tAGTtAnnn(An)) (Chambers *et al.*, 1990). Table 19.1 summarizes the primary known functions of each of the gene products,

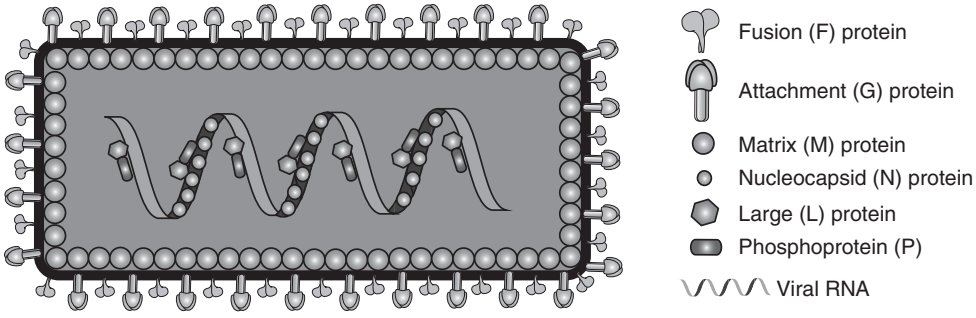

Fig. 19.1. Schematic of a PVM virion with the nucleocapsid surrounded by M protein. The viral envelope has projections from the F and G proteins. The embedded SH membrane protein is not shown.

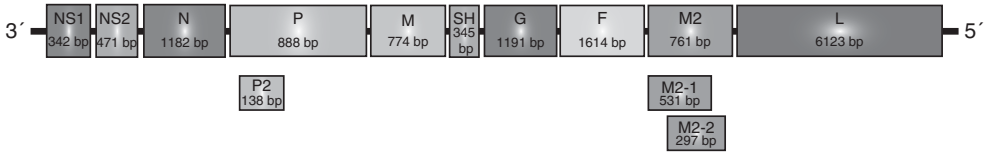

Fig. 19.2. The PVM genome shown to scale with 10 primary coding sequences and intergenic spacers. Transcript lengths for P2, M2-1 and M2-2 are shown under the genome at loci where transcription is initiated.

Table 19.1. PVM strain J3666 genome details, GenBank accession number NC_006579.

Coding region	Nucleotide length (bp)	Amino acid length	Gene product(s)	Primary function(s)
NS1	342	113	Non-structural protein-1	Both NS proteins inhibit RNA synthesis, and inhibit cellular responses to interferon
NS2	471	156	Non-structural protein-2	
N	1182	393	Nucleocapsid protein	Essential component of the polymerase complex
P	888	295	Phosphoprotein	Essential for genome replication and transcription
			P2	Function unknown
M	774	257	Matrix protein	Renders the nucleocapsid transcriptionally inactive during packaging
SH	345	114	Small hydrophobic protein	Unknown, not essential for RSV replication, not likely essential for PVM replication
G	1191	396	Attachment glycoprotein	Virus attachment
F	1614	537	Fusion glycoprotein	Cell fusion, evasion of innate immune responses
M2	531	176	M2-1	Virus RNA synthesis
	297	98	M2-2	Putative role in switching from genome replication to assembly and packaging
L	6123	2040	RNA polymerase	The major component of the RNA-dependent RNA polymerase

some of which have been extrapolated from results of basic work on human and bovine RSVs.

The non-structural proteins, NS-1 and NS-2, are at the 3′ end of the genome, and thus are transcribed in higher abundance than the other genes. Their function in the virus life cycle appears to be primarily to aid in the evasion of host interferon (IFN) responses (Heinze *et al.*, 2011). The N protein of PVM is believed to play a structural role in the formation of the helical nucleocapsid complex with close association with the viral genomic RNA. Reverse genetics experiments have demonstrated that it is an essential component of the polymerase complex. The P gene product has two open reading frames (ORFs) (Barr *et al.*, 1994), encoding the P protein and several P-like proteins each translated from internal AUG start codons within the P protein ORF. The function(s) of the P-related proteins (including P2) remain unknown, but the phosphoprotein is necessary for genome replication. The product of the L gene is the catalytic component of the RNA-dependent RNA polymerase (Thorpe and Easton, 2005). The mRNA from the M2 gene has two ORFs, encoding two proteins, M2-1 and M2-2. Studies done using the related pneumovirus, RSV, indicate that the M2-1 product is essential for genome replication, playing a role in stabilizing and enhancing the production of full-length RNA. In contrast, reverse genetics experiments using hRSV indicate that expression of M2-2 interferes with virus gene expression (Collins *et al.*, 1995, 1996; Bermingham and Collins, 1999) as it accumulates inside the infected cell. This event is thought to signal a switch from genome replication to virion assembly in preparation for release from the infected cell. The PVM G attachment protein is a type II glycoprotein with its carboxy-terminus projecting from the viral envelope, and from the membrane of the infected epithelial cell (Ling and Pringle, 1989a, 1989b). The protein product is modified extensively post-transcription to include both O- and N-glycosylations. The most substantial genetic difference between the non-pathogenic PVM strain 15 and the highly

pathogenic strain J3666 was found in the G protein, where a 35 amino acid membrane anchor was detected only in strain J3666 (Randhawa *et al*., 1995). The PVM fusion (F) protein is a type I glycoprotein, that like the G protein is easily detected on the surface of infected epithelial cells. The function of the small hydrophobic (SH) gene product is unknown, but reverse genetics experiments performed with hRSV confirm that it is not essential to virus replication. Substantial sequence variation between strains 15 and J3666 indicate a potential role for the SH gene product in pathogenesis, an area that is currently being explored (Thorpe and Easton, 2005).

19.5 Pathogenesis

19.5.1 Clinical features of infection

PVM specifically targets the epithelial mucosa of the respiratory tract, including the nose, trachea, bronchi and bronchioles, with minimal involvement of subepithelial tissue. The lung consolidation seen from PVM infection is different from that caused by other respiratory viruses such as influenza, particularly early during the illness when a distinct pattern of bronchiolitis is observed (Fig. 19.3). Intranasal challenge with PVM with as little as 10 plaque forming units/mouse results in robust virus replication with strains 15 and J3666 alike. The main difference between challenges with the two strains is the degree of morbidity. PVM J3666, even at very low inoculum, leads to severe infection. An inoculum of 100 pfu is sufficient to cause 100% mortality in Balb/c mice, and the more resistant C57Black/6 mice will die from a challenge of 300 pfu or more. Mice infected with PVM J3666 become lethargic, anorectic, hunched and ruffled. Weight loss is evident early during infection, as early as day 3 following inoculation. A systematic evaluation of lung function during sub-lethal infection with PVM J3666, as measured by whole body plethysmography, showed that lung function declined in parallel with weight loss and lung virus titre (Bonville *et al*., 2006). Interestingly, although virus was no longer detectable by day 10 post-inoculation, lung function did not return to baseline until day 28. Clinical illness from PVM strain-15 infection is dependent on the isolate of the virus used. Strain 15 Warwick describes an isolate that has been passed in tissue culture for more than two decades. Challenge with this replication-competent strain 15 virus, at inocula similar to or higher than doses used in severe illness models with the J3666 strain, causes little to no clinical illness (Domachowske *et al*., 2002). The availability of PVM strains with different

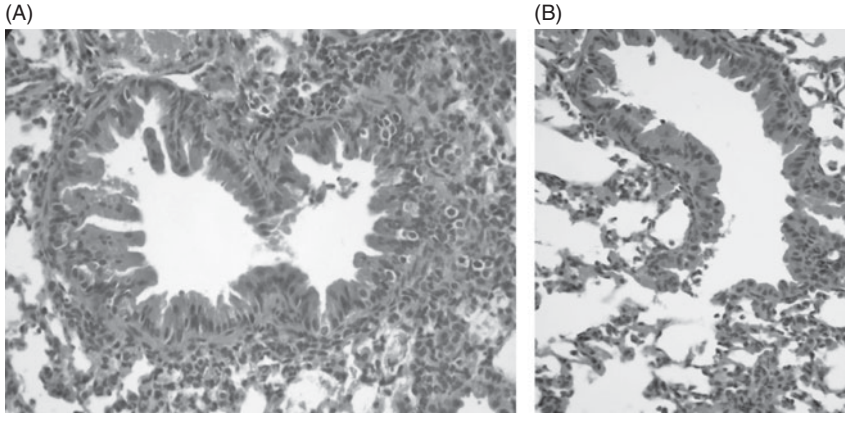

Fig. 19.3. (A) PVM-infected lung showing characteristic inflammatory bronchiolitis and pneumonitis with prominent bronchiolar hyperplasia. (B) Terminal bronchiolitis, as shown, is a consistent finding early in infection, prior to the development of panalveolitis.

virulence and the known differences in susceptibility among various inbred strains of mice have allowed further studies on immunity and pathogenesis, including testing the successful evaluation of mucosal challenge with avirulent PVM as an effective vaccine against later challenge with a lethal inoculum of PVM J3666 (Ellis *et al.*, 2007).

19.5.2 The early innate responses to PVM infection

After intranasal challenge with virulent strains of PVM, virus replication is accompanied by the influx of granulocytes. In the first days of infection, the recruitment of eosinophils is apparent, quickly to be replaced by neutrophils (Domachowske *et al.*, 2000). In experiments where antiviral agents were administered to infected mice beginning 3 days after challenge, virus clearance was more rapid, but the virus-induced inflammatory responses, including ongoing expression and release of inflammatory mediators and the presence of granulocyte influx, persisted (Domachowske *et al.*, 2001a). This innate immune response was found to be driven by the release of several mediators from the infected epithelial cells (Domachowske *et al.*, 2001b). One of the best-studied innate immune mediators known to be important in PVM disease pathogenesis is CCL3 (MIP-1α). CCL3 is one of several mediators that is expressed and released during virulent J3666 infection, but not during strain 15 infection (Domachowske *et al.*, 2002), and its lung concentrations correlated directly with clinical and physiologic measures of illness severity (Bonville *et al.*, 2006). Since CCL3 is a potent neutrophil and eosinophil chemoattractant, it is not surprising that, upon signaling blockade, far fewer granulocytes are recruited to the PVM-infected lung. Mice that are genetically deficient in CCL3 do not mount a robust granulocyte response to PVM infection (Bonville *et al.*, 2003). In addition, immunologic blockade of CCL3 signaling with anti-CCL3 antibody (Bonville *et al.*, 2003), or direct interference with its signaling pathway via blockade of its primary receptor CCR1 (Bonville *et al.*, 2004), greatly reduces granulocyte influx *in vivo*. While it might be expected that a reduction in granulocyte recruitment during PVM infection, by itself, could have an impact on disease manifestations, the ultimate natural course of infection (death) was not altered under any of the aforementioned conditions unless antiviral therapy (ribavirin) was included. If CCL3 signaling was interrupted, and antiviral therapy administered beginning on day 3 of infection, illness severity was greatly attenuated, including a 90% or more reduction in mortality (Bonville *et al.*, 2003, 2004).

The implications of these findings are that, while the development of antiviral agents for the treatment of pneumovirus infection in humans infected with hRSV (or calves that are infected with bRSV) can impact disease pathogenesis, to do so efforts must consider blocking virus-induced inflammatory pathways contemporaneously. As discussed, one successful way in which this can be accomplished in mice is through interference with the CCL3 signaling pathway. This observation led to a series of studies designed to determine whether other known anti-inflammatory interventions might have similar benefits. One strategy that was considered was the introduction of glucocorticoids during the course of PVM infection. Given the protean anti-inflammatory effects of glucocorticoids, it was logical to consider the combined use of antiviral agents with glucocorticoids in an attempt achieve therapeutic benefit. Somewhat unexpectedly, PVM-challenge studies showed that treatment with glucocorticoids was counterproductive, resulting in little effect on virus-induced inflammation, higher PVM lung titres and more rapid lethality (Domachowske *et al.*, 2001a). *In vitro* studies confirmed that pneumovirus-infected cells are resistant to the usual anti-inflammatory effects of glucocorticoids, particularly with regard to the induction and release of chemoattractant cytokines (Bonville *et al.*, 2001).

While the contributions of CCL3 to PVM disease is the best studied of the

chemokines, several other innate inflammatory mediators are also known to contribute to the pathogenesis of the infection, including CCL2 (MCP-1) and CXCL2 (MIP-2). Lung concentrations of both of these mediators have been shown to correlate with the objective measures of lung dysfunction and clinical illness scores (Bonville *et al.*, 2006). Furthermore, PVM infection, like most respiratory viral infections, also induces pulmonary expression of IFN-β along with the resulting extensive array of IFN response genes (Domachowske *et al.*, 2002). In the genetic absence of type two IFN receptor, PVM-infected mice have altered inflammatory responses to infection with distinctive lung pathology resulting from preferential expression of CCL24 (eotaxin-2), thymus and activation-regulated chemokine (TARC), and mouse eosinophil-associated ribonuclease 11 (Garvey *et al.*, 2005), further emphasizing an important regulatory role of IFN signaling in PVM-associated inflammation.

19.5.3 Adaptive cellular responses to PVM infection

Following virulent strain PVM challenge, clinical symptoms and pulmonary inflammation develop rapidly, with mice becoming moribund a week or less after challenge, so the primary focus of research on PVM pathogenesis has been on granulocyte recruitment and innate immune responses. Use of less virulent strains, and very low inocula of strain J3666, has facilitated the development of long-term survival models so that later cell-mediated immune response to infection could be explored. Claasen *et al.* (2005) defined and detected three PVM specific $CD8^+$ T-cells recognizing epitopes from PVM proteins P, M and F in the lungs of infected mice as early as day 8 post-challenge. The finding that these cells were largely non-cytokine producing and silent in their expression of IFN supports the ongoing hypothesis that pneumoviruses have the capacity to subvert the normal antiviral host IFN responses.

19.5.4 PVM infection at the extremes of age

In the laboratory, age at PVM infection also contributes to illness manifestations and the observed severity of the inflammatory response. In one study of neonatal mice, clinical and biochemical responses to PVM infection appeared to develop and evolve during the first month of life (Bonville *et al.*, 2010). The study evaluated infection in mice at 7, 14, 21 and 28 days of age, and despite similar virus replication kinetics in all age groups, 7-day-old mice had significantly blunted levels of CCL2, CCL3, CCL5, CCL8, CCL11, CXCL9, CXCL10 and IFN-γ transcripts compared with their older counterparts. Protein levels of the aforementioned mediators detected in lung homogenates were consistent with transcript levels, with lower chemokine concentrations measured from the 7-day-old mice compared with the other groups. In a study on the other extreme of age, PVM infection of seronegative young adult mice (8 weeks) was compared with older age cohorts, including an advanced age group at 78 weeks. Among all age groups evaluated, the kinetics of PVM replication was similar, but older mice demonstrated diminished local production of several proinflammatory mediators, including, CCL2, CCL3 and IFN-γ, along with diminished recruitment of granulocytes to the lung tissue (Bonville *et al.*, 2007). This was most pronounced in the 'geriatric' mice at 78 weeks of age. The observations from studies done at the extremes of ages aid in our further understanding of the development of innate immunity to pneumovirus during early development as well as further characterization of the natural course of innate immune senescence during ageing.

19.6 Culturing and Identifying PVM in the Laboratory

Through the use of traditional cell culture, PVM can be isolated and amplified for experimental purposes. Traditionally, the primate BS-C-1 epithelial cell line has been

used for performing quantitative plaque assays to titre virus. To a limited extent, other cell lines also support PVM replication, including the rodent epithelial lines L2, LA4 and RLE, murine macrophage cell lines RAW 267.4 and J774A.1, murine T3T fibroblasts and human A549 and HEp-2 cells (Dyer *et al.*, 2007). Mink lung epithelial cells are also permissive to infection with PVM. Virus replication can be confirmed by molecular methods or by immunofluorescence and/or immunohistochemical staining (Carthew and Sparrow, 1980). More recently, the development of reverse-transcriptase polymerase chain reaction (RT-PCR) has allowed for rapid testing and confirmation of infection in research colonies.

Standard surveillance for murine pathogens now includes serologic testing for PVM, usually by enzyme-linked immunosorbent assay (ELISA). The uninvited detection of PVM in a mouse colony is a nuisance since eradication is difficult once the virus is detected. Large animal facilities that house transgenic mice, particularly those that are immunodeficient, must be especially vigilant to detect PVM early, and institute eradication plans immediately, because PVM can rapidly decimate highly susceptible, often very valuable research colonies (Richter *et al.*, 1988; Weir *et al.*, 1988). Screening can be performed on a regular basis, or when introduction of PVM is considered because of illness in the colony. Molecular assays that are available commercially include both traditional and quantitative RT-PCR. Serum from sentinel or sick animals can also be tested for the presence of PVM-specific antibody.

19.7 Control of Infection

PVM was highly prevalent in mouse colonies before routine surveillance programmes, but presently, given careful infection control procedures, colony contamination with PVM is uncommon. A large-scale study performed between 2004 and 2007 showed that PVM is not a common microbial contaminant of mouse colonies, only being detected in 2/25 mouse and 2/16 rat colonies screened (Liang *et al.*, 2009). Prevention is key, since eradication of inadvertent introduction of the pathogen can be difficult, time consuming and expensive. Mice that are going to be infected with PVM and used for research purposes should be housed separately from areas where rodent pathogens are not being explored experimentally. When a colony of rodents becomes infected with PVM, standard procedures, including quarantine and temporary cessation of breeding efforts should be initiated.

19.8 Modelling Human Pneumovirus Disease with PVM

PVM infection in the wild is commonplace, but has little to no negative impact on the ecosystem or on human interests such as agriculture or potential for transmission to humans. Instead, the recent resurgence of interest in PVM relates specifically to its potential to unveil novel concepts in the study of pneumovirology in the larger context, including better understanding of human and bovine respiratory syncytial virus infection. The strengths and limitations of the PVM model in this regard were recently reviewed (Domachowske *et al.*, 2004). In general, studies on the pathogenesis of PVM infection have demonstrated impressive parallels to those found in studies of the pathogenesis of human RSV disease. In particular, studies of the innate immune responses to these viruses during infection of their natural hosts demonstrate marked similarities in patterns, and in biomarkers that are associated with severe illness. Striking similarities have also been observed on histopathology, especially for the more severe forms of infection. These parallels have identified PVM infection of mice as an excellent model for the study of pneumovirus pathogenesis, and responses to treatment interventions.

Awareness of the potential for PVM to be introduced into rodent research housing facilities remains important for all investigators who use rodents for their research. The use of mouse and rat models for the

study of human disease pathogenesis is commonplace. The observation that mild and/or subclinical PVM infection changes pulmonary gene expression, even in the absence of obvious clinical illness, underscores the importance of regular surveillance of research colonies for the presence of virus. The potential detrimental effects of intercurrent infection, even one that is clinically silent, on the outcomes of comparative immunological research in other fields cannot be overstated.

References

Anh, D.B.T., Faisca, P. and Desmecht, D.J.M. (2006) Differential resistance/susceptibility patterns to pneumovirus infection among inbred mouse strains. *American Journal of Physiology Lung Cellular and Molecular Physiology* 291, L426–L435.

Barr, J., Chambers, P., Harriott, P., Pringle, C.R. and Easton, A.J. (1994) Sequence of the phosphoprotein gene of pneumonia virus of mice: expression of multiple proteins from 2 overlapping reading frames. *Journal of Virology* 68, 5330–5334.

Bermingham, A. and Collins, P.L. (1999) The M2-2 protein of human respiratory syncytial virus is a regulatory factor involved in the balance between RNA replication and transcription. *Proceedings of the National Academy of Sciences USA* 96, 11,259–11,264.

Berthiaume, L., Joncas, J. and Pavilanis, V. (1974) Comparative structure, morphogenesis and biological characteristics of the respiratory syncytial (RS) virus and the pneumonia virus of mice (PVM). *Archiv für die gesamte Virusforschung* 45, 39–51.

Bonville, C.A., Mehta, P., Krilov, L., Rosenberg, H.F. and Domachowske, J.B. (2001) Epithelial cells infected with respiratory syncytial virus are resistant to the anti-inflammatory effects of hydrocortisone. *Cellular Immunology* 213, 134–140.

Bonville, C.A., Easton, A.J., Rosenberg, H.F. and Domachowske, J.B. (2003) Altered pathogenesis of severe pneumovirus infection in response to combined anti-viral and specific immunomodulatory agents. *Journal of Virology* 77, 1237–1244.

Bonville, C.A., Lau, V.K., deLeon, J.M., Gao, J.L., Easton, A.J., Rosenberg, H.F. and Domachowske, J.B. (2004) Functional antagonism of the chemokine receptor CCR1 is an effective anti-inflammatory strategy in severe pneumovirus infection in vivo. *Journal of Virology* 78, 7984–7989.

Bonville, C.A., Bennett, N.J., Koehnlein, M., Haines, D.M., Ellis, J.A., Del Vecchio, A.M., Rosenberg, H.F. and Domachowske, J.B. (2006) Respiratory dysfunction and proinflammatory chemokines in the pneumonia virus of mice (PVM) model of viral bronchiolitis. *Virology* 69, 53–59.

Bonville, C.A., Bennett, N.J., Percopo, C.M., Branigan, P.J., Del Vecchio, A.M., Rosenberg, H.F. and Domachowske, J.B. (2007) Diminished inflammatory responses to natural pneumovirus infection among older mice. *Virology* 368, 182–190.

Bonville, C.A., Ptaschinski, C., Percopo, C.M., Rosenberg, H.F. and Domachowske, J.B. (2010) Inflammatory responses to acute pneumovirus infection in neonatal mice. *Journal of Virology* 7, 320.

Brock, L.G., Karron, R.A., Krempl, C.D., Collins, P.L. and Buchholz, U.J. (2012) Evaluation of pneumonia virus of mice as a possible human pathogen. *Journal of Virology* 86, 5829–5843.

Carthew, P. and Sparrow, S. (1980) A comparison in germ-free mice of pathogenesis of Sendai virus and mouse pneumonia virus infections. *Journal of Pathology* 30, 153–158.

Chambers, P., Matthews, D.A., Pringle, C.R. and Easton, A.J. (1990) The nucleotide sequences of intergenic regions between nine genes of pneumonia virus of mice establish the physical order of these genes in the viral genome. *Virus Research* 18, 263–270.

Claassen, E.A., van der Kant, P.A., Rychnavska, Z.S., van Bleek, G.M., Easton, A.J. and van der Most, R.G. (2005) Activation and inactivation of antiviral CD8 T cell responses during murine pneumovirus infection. *Journal of Immunology* 175, 6597–6604.

Collins, P.L., Hill, M.G., Camargo, E., Grosfeld, H., Chanock, R.M. and Murphy, B.R. (1995) Production of infectious human respiratory syncytial virus from cloned cDNA confirms an essential role for the transcription elongation factor from the 5′ proximal open reading frame of the M2 mRNA in gene expression and provides a capability for vaccine development. *Proceedings of the National Academy of Sciences USA* 92, 11,563–11,567.

Collins, P.L., Hill, M.G., Cristina, J. and Grosfeld, H. (1996) Transcription elongation factor of respiratory syncytial virus, a nonsegmented negative-strand RNA virus. *Proceedings of the National Academy of Sciences USA* 93, 81–85.

Compans, R.W., Harter, D.H. and Choppin, P.W. (1967) Studies of pneumonia virus of mice (PVM) in cell culture. II: Structure and morphogenesis of the virus particle. *Journal of Experimental Medicine* 126, 267–277.

Cook, P.M., Eglin, R.P. and Easton, A.J. (1998) Pathogenesis of pneumovirus infections in mice: detection of pneumonia virus of mice and human respiratory syncytial virus mRNA in lungs of infected mice by *in situ* hybridisation. *Journal of General Virology* 79, 2411–2417.

Domachowske, J.B., Bonville, C.A., Gao, J.L., Murphy, P.M., Easton, A.J. and Rosenberg, H.F. (2000) The chemokine macrophage inflammatory protein-1 alpha and its receptor CCR1 control pulmonary inflammation and antiviral host defense in paramyxovirus infection. *Journal of Immunology* 165, 2677–2682.

Domachowske, J.B., Bonville, C.A., Ali-Ahmad, D., Dyer, K.D., Easton, A.J. and Rosenberg, H.F. (2001a) Glucocorticoid administration accelerates mortality of pneumovirus-infected mice. *Journal of Infectious Diseases* 184, 1518–1523.

Domachowske, J.B., Bonville, C.A. and Rosenberg, H.F. (2001b) Gene expression in epithelial cells in response to pneumovirus infection. *Respiratory Research* 2, 225–233.

Domachowske, J.B., Bonville, C.A., Easton, A.J. and Rosenberg, H.F. (2002) Differential expression of proinflammatory cytokine genes in vivo in response to pathogenic and nonpathogenic pneumovirus infection. *Journal of Infectious Diseases* 186, 8–14.

Domachowske, J.B., Bonville, C.A. and Rosenberg, H.F. (2004) Animal models for studying respiratory syncytial virus infection and its long-term effects on lung function. *Pediatric Infectious Disease Journal* 23, S228–234.

Dyer, K.D., Schellens, I.M., Bonville, C.A., Martin, B.V., Domachowske, J.B. and Rosenberg, H.F. (2007) Efficient replication of pneumonia virus of mice (PVM) in a mouse macrophage cell line. *Virology Journal* 4, 48.

Easton, A.J., Domachowske, J.B. and Rosenberg, H.F. (2004) Animal pneumoviruses: molecular genetics and pathogenesis. *Clinical Microbiology Reviews* 17, 390–412.

Eleraky, N.Z., Kania, S.A., Evermann, J.F. and Potgieter, L.N. (2003) Comparison of targeting F and G protein genes to detect bovine and ovine respiratory syncytial viruses. *Journal of Veterinary Diagnostic Investigation* 15, 277–280.

Ellis, J.A., Martin, B.V., Walder, C., Dyer, K.D., Domachowske, J.B. and Rosenberg, H.F. (2007) Mucosal inoculation with an attenuated mouse pneumovirus strain protects against virulent challenge in wild type and interferon receptor deficient mice. *Vaccine* 25, 1085–1095.

Gannon, J. and Carthew, P. (1980) Prevalence of indigenous viruses in laboratory animal colonies in the United Kingdom 1978–1979. *Laboratory Animals* 14, 309–311.

Garvey, T.L., Dyer, K.D., Ellis, J.A., Bonville, C.A., Easton, A.J., Domachowske, J.B. and Rosenberg, H.F. (2005) Inflammatory responses to pneumovirus infection in interferon-alpha/beta receptor-deleted mice. *Journal of Immunology* 175, 4735–4744.

Heinze, B., Frey, S., Mordstein, M., Schmitt-Gräff, A., Ehl, S., Buchholz, U.J., Collins, P.L., Staeheli, P. and Krempl, C.D. (2011) Both nonstructural proteins NS1 and NS2 of pneumonia virus of mice are inhibitors of the interferon type I and type III responses in vivo. *Journal of Virology* 85, 4071–4084.

Horsfall, F.L. and Curnen, E.C. (1946) Studies on pneumonia virus of mice (PVM). II. Immunological evidence of latent infection with the virus in numerous mammalian species. *Journal of Experimental Medicine* 83, 43–64.

Horsfall, F.L. and Hahn, R.G. (1939) A pneumonia virus of Swiss mice. *Proceedings of the Society for Experimental Biology and Medicine* 40, 684–686.

Horsfall, F.L. and Hahn, R.G. (1940) A latent virus in normal mice capable of producing pneumonia in its natural host. *Journal of Experimental Medicine* 71, 391–408.

Intisar, K.S., Ali, Y.H., Khalafalla, A.I., Rahman, M.E. and Amin, A.S. (2010) Respiratory syncytial virus infection of camels (*Camelus dromedaries*). *Acta Tropica* 113, 129–133.

Kagiyama, N., Takakura, A. and Itoh, T. (1986) A serological survey on 15 murine pathogens in mice and rats. *Jikken Dobutsu* 35, 531–536.

Kaplan, C., Healing, T.D., Evans, N., Healing, L. and Prior, A. (1980) Evidence of infection by viruses in small British field rodents. *Journal of Hygiene – Cambridge* 84, 285–294.

Krempl, C.D. and Collins, P.L. (2004) Reevaluation of the virulence of prototypic strain 15 of pneumonia virus of mice. *Journal of Virology* 78, 13,362–13,365.

Krempl, C.D., Lamirande, E.W. and Collins, P.L. (2005) Complete sequence of the RNA genome of pneumonia virus of mice (PVM). *Virus Genes* 30, 237–249.

Lehmkuhl, H.D., Smith, M.H. and Cutlip, R.C. (1980) Morphogenesis and structure of caprine respiratory syncytial virus. *Archives of Virology* 65, 269–276.

Liang, C.T., Shih, A., Chang, Y.H., Liu, C.W., Lee, Y.T., Hsieh, W.C., *et al*. (2009) Microbial contaminations of laboratory mice and rats in Taiwan from 2004 to 2007. *Journal of the American Association of Laboratory Animal Science* 48, 381–386.

Ling, R. and Pringle, C.R. (1989a) Polypeptides of pneumonia virus of mice. I: Immunological cross-reactions and post-translational modifications. *Journal of General Virology* 70, 1427–1440.

Ling, R. and Pringle, C.R. (1989b) Polypeptides of pneumonia virus of mice. II: Characterisation of the glycoproteins. *Journal of General Virology* 70, 1441–1452.

Parker, J.C., Tennant, R.W. and Ward, T.G. (1966) Prevalence of viruses in mouse colonies. *National Cancer Institute Monthly* 20, 25–36.

Pringle, C.R. and Eglin, R.P. (1986) Murine pneumonia virus: seroepidemiological evidence of widespread human infection. *Journal General Virology* 67, 975–982.

Randhawa, J.S., Chambers, P., Pringle, C.R. and Easton, A.J. (1995) Nucleotide sequences of the genes encoding the putative attachment glycoprotein (G) of mouse and tissue culture-passaged strains of pneumonia virus of mice. *Virology* 207, 240–245.

Renshaw, R.W., Zylich, N.C., Laverack, M.A., Glaser, A.L. and Dubovi, E.J. (2010) Pneumovirus in dogs with acute respiratory disease. *Emerging Infectious Diseases* 16, 993–995.

Richter, C.B., Thigpen, J.E., Richter, C.S. and Mackenzie, J.M. Jr (1988) Fatal pneumonia with terminal emaciation in nude mice caused by pneumonia virus of mice. *Laboratory Animal Sciences* 38, 255–261.

Thorpe, L.C. and Easton, A.J. (2005) Genome sequence of the non-pathogenic strain 15 of pneumonia virus of mice and comparison with the genome of the pathogenic strain J3666. *Journal of General Virology* 86, 159–169.

Weir, E.C., Brownstein, D.G., Smith, A.L. and Johnson, E.A. (1988) Respiratory disease and wasting in athymic mice infected with pneumonia virus of mice. *Laboratory Animal Sciences* 38, 133–137.

20 Infectious Hematopoietic Necrosis Virus

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20.1 Introduction

Aquaculture is a major industry with an average annual growth rate of approximately 7% and globally is the fastest growing sector in animal-based food production. However, the biggest threat to sustainable growth of fish aquaculture is viral disease. One of the most important of these fish viral diseases is infectious haematopoietic necrosis (IHN), which is a disease of salmonid fish caused by the infectious haematopoietic necrosis virus (IHNV) (Walker and Winton, 2010). IHN has widespread geographic distribution and seriously impacts both farmed and native salmonid stocks. IHN was first described in the 1950s in sockeye (*Oncorhynchus nerka*) and Chinook (*O. tshawytscha*) salmon, and then in rainbow trout (*O. mykiss*) in 1967 (Amend and Smith, 1975). Acute clinical infections are most prevalent in fry and juvenile fish. IHNV is one of the three aquatic Rhabdoviruses listed as reportable by the World Organization for Animal Health (OIE) and is considered to be a constraint to the expansion of salmonid aquaculture in the USA (Bootland and Leong, 2011).

The major impact of IHNV is on farms that rear young rainbow trout or salmon where cumulative mortality can reach 90–100% (Bergmann *et al*., 2003; Purcell *et al*., 2012a). In addition to direct losses due to mortality, the disease has a negative impact on the breeding of endangered salmon stocks, causes restrictions on the movement of IHNV-infected fish or survivors, and necessitates the destruction of infected stocks as a containment strategy. It was estimated that IHN caused a minimum of US$3 million loss to the Idaho (USA) trout industry alone (Congleton, 1988). This is due to direct mortality, decreased fish production levels and deformities that occur in the survivors. In Canada, between 2001 and 2003, IHN epizootics caused CAN$250 million loss to the Atlantic salmon industry (MacKinnon *et al*., 2008). As recently as May 2012, an IHN outbreak occurred in a salmon farm off Bainbridge Island, Washington, resulting in the removal of all fish and a significant loss to the USA salmon industry (http://tinyurl.com/c2oalmr).

20.2 Host Range and Clinical Signs

IHNV infects a broad range of salmonids including rainbow trout/steelhead (*O. mykiss*), brook trout (*Salvelinus fontinalis*), lake trout (*S. namaycush*), cutthroat trout (*O. clarkii*), brown trout (*Salmo trutta*), Atlantic salmon (*Salmo salar*), chinook salmon (*O. tshawytscha*), sockeye/kokanee salmon (*O. nerka*), chum

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salmon (*O. keta*), cherry salmon/masou salmon/Yamame trout (*O. masou*), biwa salmon (*O. masou rhoduris*), amago salmon (*O. rhodurus*), coho salmon (*O. kisutch*), Japanese charr (*S. leucomaenis*), Arctic charr (*S. alpinus*), Arctic grayling (*Thymallus arcticus*) and mountain whitefish (*Prosopium williamsoni*). The susceptibility to IHNV can vary greatly from species to species and between strains within a species. IHNV has also been isolated from European eel (*Anguilla anguilla*) (Bergmann *et al.*, 2003). Through laboratory studies, the virus was also shown to infect other related salmonids, such as gilt-head bream (*Sparus aurata*), turbot (*Scophthalmus maximus*), sea bass (*Dicentrarchus labrax*), northern pike (*Esox lucius*) and pile surfperch (*Rhacochilus vacca*). Research is ongoing to determine if other hosts could be carriers of this virus to help explain viral distribution and pinpoint the potential cause of epizootics. For example, a recent study examined the ability of the Pacific lamprey to act as a host and found that it did not develop clinical signs or accumulate virus higher than the amount originally injected (Kurath *et al.*, 2012).

IHNV is thought to have originated in western North America. However, the current geographic distribution of IHNV is concentrated in many of the major fish-producing nations of the world. It is endemic in the Pacific Northwest (USA and Canada) watersheds that contain salmonids. It is also endemic in Japan as well as being found in several countries in Asia and Europe. It was first described in Europe in France and Italy in 1987, then in Germany in 1992 (Bergmann *et al.*, 2003). A recent survey from spawning chum salmon in Korea showed that IHNV was present in both adults and fry; the prevalence has varied from year to year since the survey began in 2006 (Jeon *et al.*, 2012).

The first sign of an acute IHN outbreak is a sudden increase in fish mortality. However, some fish may die without showing any of the clinical signs associated with IHN. Clinical signs include distended abdomen, haemorrhaging, anaemia, pale gills, exophthalmia (pop-eye) and skin darkening (Fig. 20.1). This can be accompanied by a pale liver, spleen and kidney due to the anaemia. Ascites can accumulate and the stomach can be filled with watery fluid (red to yellow) rather than food. This can be accompanied by petechial haemorrhages in the visceral mesenteries, swim bladder, adipose tissue, peritoneum, meninges and pericardium (Bootland and Leong, 2011). The disease can progress to a lethal necrosis of the haematopoietic tissues of the kidney and spleen. In acute viral epizootic disease, a generalized viraemia with associated necrosis can be observed in all tissues – this is thought to be caused by the virus passing from the gills to the circulatory system, which then distributes the virus to all organs and tissues. Death probably is due to renal failure caused by electrolyte imbalance (Rodríguez Saint-Jean *et al.*, 2012).

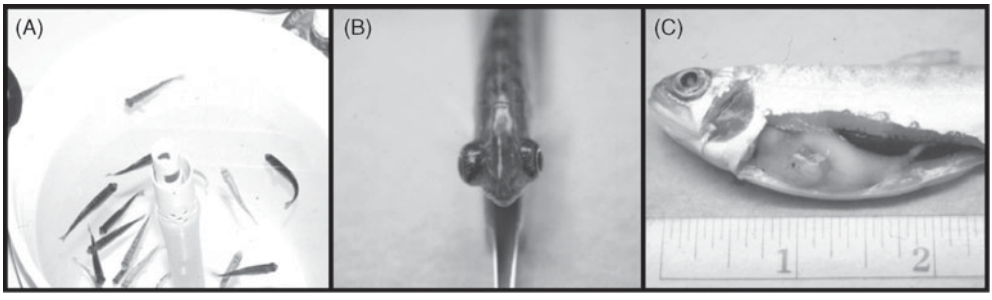

Fig. 20.1. Gross pathological symptoms of IHN disease in rainbow trout. Panel A shows IHNV-infected fish displaying darkened body coloration compared to lighter naïve fish. Panel B shows severe exophthalmia or popeye symptom, which is a classic sign of IHN. Panel C shows the pale gills and liver that are clinical signs of the anaemia caused by IHN.

Histological manifestations of IHN include destruction of the haematopoietic tissue. The stained section of a normal kidney is shown in comparison to the kidney of an IHN-infected animal in Fig. 20.2 (panels A and B, respectively). The total destruction of the haematopoietic tissue is an obvious clinical sign of this disease.

20.3 Virus Transmission

IHNV can be transmitted both horizontally through fish-to-fish contact, and vertically with virus associated with the egg. However, the disease is transmitted mostly by fish to fish through direct contact or through water in which virus was shed by infected fish through faeces, urine, sexual fluids or external mucus. Vertical transmission through eggs is also possible, although its presence inside the egg itself is not fully demonstrated, and somewhat controversial. The risk of egg-associated transmission of the virus can be significantly reduced through the use of iodophor disinfectants and virus-free water supplies.

20.4 Genome Organization and Gene Expression

The aetiologic agent of IHN is a virus. The IHNV virions are bullet shaped, measuring approximately 110 nm in length and 70 nm in diameter (Fig. 20.3A). IHNV is the type species in the genus *Novirhabdovirus* within the family *Rhabdoviridae*. Other fish *Novihabdovirus* species are viral haemorrhagic septicaemia virus (VHSV), hirame rhabdovirus (HIRRV), snakehead rhabdovirus (SHRV), eel virus B12 (EV-B12) and eel virus C26 (EV-C26). The genome of IHNV contains a single-stranded, negative-sense RNA of approximately 11.1 kb (Morzunov *et al.*, 1995; Schutze *et al.*, 1995). There are six genes in the viral genome that are, in the 3′ to 5′ order: nucleocapsid (N), polymerase-associated phosphoprotein (P), matrix (M), surface glycoprotein (G), nonvirion protein (NV) and virus polymerase (L) (Schutze *et al.*, 1995). These are shown in the diagram in Fig. 20.3A. The presence of the NV protein is a distinguishing feature of the *Novirhabdovirus* genus within the *Rhabdovirus* family (Kurath *et al.*, 1997). There is a 60-nt leader sequence on the 3′-end and a 101-nt trailer sequence on the 5′-end of the genome (Fig. 20.3B). The non-coding sequences at either end of the viral genome are complementary to each other and are likely to form a panhandled structure for priming the initiation of RNA synthesis similar to other members of the family *Rhabdoviridae* (Banerjee and Barik, 1992).

In IHNV-infected cell lines, all six virally encoded genes have been identified. Among these genes, the N protein gene (N gene) is expressed 2–3 hours post-infection and is the most abundant protein produced

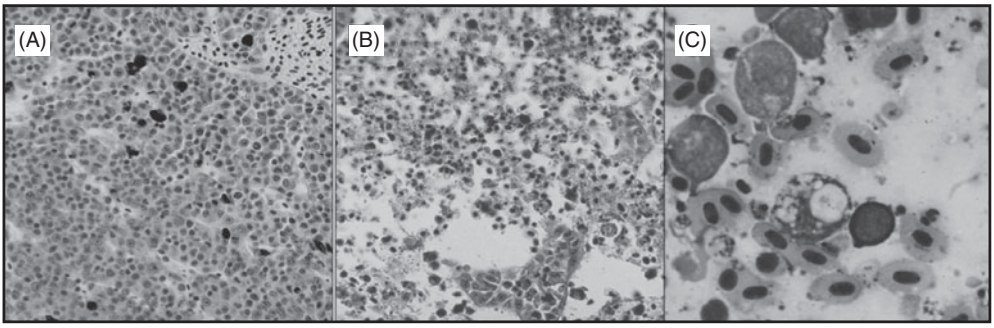

Fig. 20.2. Histological manifestations of IHN disease in rainbow trout. Panel A is a section of normal kidney. Panel B is a section of kidney from IHN-infected fish. Note total destruction of the haematopoietic tissue. Panel C is anterior kidney organ imprint stained with DiffQuik. Note the 'foamy' macrophage in the centre of the slide and necrotic debris.

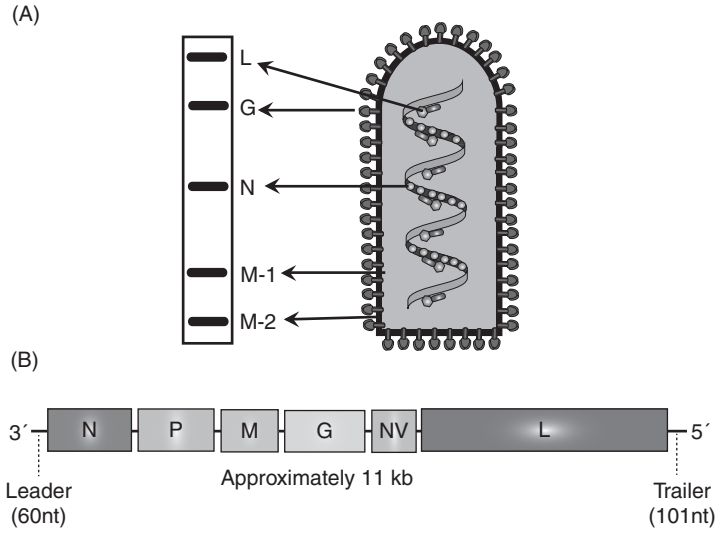

Fig. 20.3. (A) A schematic representation of the structure and protein components of IHNV. The IHNV virus contains five structural proteins: L (150–225.2 kDa), G (67–70 kDa), N (40.5–44 kDa), P or M1 (22.5–27 kDa) and M or M2 (17.5–21.8 kDa). The SDS gel is schematically represented on the left with arrows pointing to the location of the viral proteins. (B) A schematic representation of the genome organization of IHNV. IHNV genome contains a negative-sense, single-stranded RNA virus of approximately 11 kb. The location of different genes in the genome in the 3′ to 5′ order are N, P, M, G, NV and L. There are untranslated region on both 5′- and 3′-end of the genome.

during an IHNV infection owing to the presence at the 3′-end of the genome (Kurath and Leong, 1985; Leong and Kurath, 2010). Therefore, the N gene is a good target for early detection of IHNV infection. Compared with the N gene, the P and M genes are expressed later, at 6–7 hours post-infection, followed by the G (at 9–10 hours) and L (at 15 hours) genes, respectively. The N protein is present in the mature virion and remains tightly associated with the viral genome (Gilmore and Leong, 1988). During the late phase of virus infection, the N gene is highly expressed, while its corresponding protein has been shown to play a crucial role in regulating the balance between viral transcription and replication. This N protein contains 413 amino acids with a molecular mass of 40.5–44 kDa (Fig. 20.3A). Both the N- and C-terminals of the N protein contain highly hydrophilic residues, whereas the middle third of the protein contains hydrophobic residues. During the late phase of IHNV infection, the N protein binds to the nascent RNA transcript and prevents recognition of the mRNA transcription signals by the viral polymerase protein. This inhibition results in formation of a full-length plus-strand template, which is used for synthesis of the progeny minus-strand genome (Biacchesi *et al.*, 2000a, 2000b).

The P protein, previously called the M1 protein, is a highly basic (pI = 8.4) and phosphorylated protein with unknown function. The basic nature of the IHNV P protein is in sharp contrast to the homologous phosphoprotein of mammalian rhabdovirus, rabies virus and vesicular stomatitis virus (VSV). The IHNV P protein contains 231 amino acids with a molecular mass of 25.6 kDa. Sequence analysis of the P genes of different IHNV strains revealed that the P gene might also contain a small ORF encoding a highly basic, arginine-rich protein with 42 amino acids with a molecular mass of 4.8 kDa and an estimated pI of 10.1. However, this putative protein has not been identified in IHNV-infected cells, although a similar small protein encoded by an overlapping ORF has been reported in

other rhabdoviruses such as vesicular stomatitis virus and rabies virus.

Like the P protein, the M protein is also a highly basic protein (pI = 10.08) that contains a number of basic amino acids at the N-terminal end of the protein that are conserved among the homologous matrix proteins of other fish rhabdoviruses, including VHSV and HIRRV. The M protein has been shown to induce apoptosis using *in vitro* cell culture assays when transfected with plasmid DNA containing the M gene (Chiou *et al.*, 2000). However, the molecular pathways leading to apoptosis with IHNV-infected fish *in vivo* have not been determined.

The G gene in the IHNV genome encodes a G protein that forms a part of the structural protein in the mature virion. It is a membrane-associated protein that forms spike-like projections on the surface of the mature virion (Fig. 20.3A) and antibodies targeted to the G protein neutralize viral infectivity (McAllister and Wagner, 1975; Engelking and Leong, 1989). The predicted amino acid sequence of the IHNV G gene contains 508 residues with a molecular mass of 67–70 kDa (Fig. 20.3A). Both the N- and C-terminal regions contain hydrophobic domains. The N-terminal 20 amino acid residues represent a signal sequence, whereas the C-terminal hydrophobic region is likely to represent a transmembrane domain. A comparison of the predicted amino acid sequence of the IHNV G protein with that of other rhabdoviruses identified a number of conserved cysteine and proline residues between IHNV, VSV and rabies. Unlike the N- and C-terminal regions, the middle of the G-protein (also called mid-G; amino acid residues 365–430) is very hydrophilic and highly variable among different IHNV isolates, which makes the mid-G section of the G-protein a good region for phylogenetic analysis (Troyer *et al.*, 2000; Garver *et al.*, 2003; Kurath *et al.*, 2003).

Epidemiological studies of North American IHNV isolates using mid-G protein sequences revealed three major genetic subgroups, designated as U, M, and L, based on the prevalence of the isolates in the upper (U), middle (M), and lower (L) parts of the IHNV geographic range (Troyer *et al.*, 2000; Garver *et al.*, 2003; Kurath *et al.*, 2003). The U genogroup is the most prevalent in Alaska and British Columbia. However, it is also found in fish in coastal Washington, and the Columbia River basin, including Washington, Oregon and Idaho. The M genogroup occurs in the Columbia and Snake River basins, and recently in Washington coastal watersheds. In contrast, the L genogroup occurs in California and on the southern coast of Oregon. Although IHNV isolates belonging to each genogroup are capable of infecting multiple host species, there is a general trend in host-specificity among IHNV isolates in North America. The IHNV isolates from the U genogroup are highly virulent to sockeye salmon (*O. nerka*) and M genogroups causes low mortality; in rainbow trout (*O. mykiss*), M genogroups are highly virulent and U genogroups causes low mortality (LaPatra *et al.*, 1990).

The NV ('nonvirion') gene that encodes a protein of 111 amino acids is located between the G and L genes and is highly conserved among some of the fish rhabdoviruses, IHNV, VHSV and HIRRV. The NV gene is present only among the rhabdoviruses infecting fish; this distinction led to the creation of a new taxonomic genus, *Novirhabdovirus* (van Regenmortel *et al.*, 2000). The NV protein does not show significant similarity to other protein sequences in the GenBank database. The function of this protein was unknown until recently, when it was shown that a recombinant IHNV knockout with no NV gene was severely impaired for growth in cell culture and had reduced pathogenicity in rainbow trout (Thoulouze *et al.*, 2004; Biacchesi, 2011). These two studies clearly showed that the NV protein is needed for optimal growth of the virus. However, for another *Novirhabdovirus* species, snakehead rhabdovirus (SHRV), the NV protein was found to be non-essential for virus replication (Alonso *et al.*, 2004). It has been speculated that the NV protein is perhaps essential for cold-water fish Novirhabdoviruses, such as IHNV, but not for warm-water fish Novirhabdoviruses, such as SHRV, reflecting temperature adaptation of IHNV versus

SHRV to infect different hosts. It would be interesting to determine the effect of replacing IHNV NV gene with SHRV NV gene and vice versa to evaluate the complementation of the NV protein on virus phenotype, pathogenicity and host adaptation using a reverse genetics approach (Biacchesi, 2011). Further insight into the role of the NV protein in IHNV pathogenicity has been elucidated by Choi *et al.* (2011). These authors demonstrated that the NV protein supports the growth of IHNV through inhibition of the induction of host IFN systems. The 111 amino acid-containing NV protein contains a nuclear localization signal (^{32}EGDL^{35}), which plays an important role in the inhibitory activity of NV.

The L gene encodes a protein of 1986 amino acids with a predicted molecular mass of approximately 225 kDa, and shows similarity to the RNA-dependent RNA polymerase (RdRp) genes of other rhabdoviruses. The IHNV RdRp contains six conserved domains similar to other unsegmented, negative-strand RNA viruses (Poch *et al.*, 1990). Phylogenetic analysis based on the L gene revealed that Novirhabdoviruses are more related to plant rhabdoviruses than to rhabdoviruses that infect terrestrial animals (Bourhy *et al.*, 2005).

20.5 Viral Pathogenesis

After waterborne exposure, IHNV enters a fish through the gills, skin, fin bases, mouth and oesophagus/cardiac/stomach regions and then the virus replicates in epidermal cells (Mulcahy *et al.*, 1982; Yamamoto *et al.*, 1990; Helmick *et al.*, 1995; Arkush *et al.*, 2004; Foott *et al.*, 2006; Harmache *et al.*, 2006). In rainbow trout, upon viral entry, transient replication of the virus occurs in fin bases, gills and skin before spreading to internal organs, although some researchers reported that skin did not serve as a portal for entry of the virus (Brudeseth *et al.*, 2002). Harmache *et al.* (2006) used a recombinant infectious IHNV expressing the *Renilla* luciferase gene and bioluminescent imaging to show that the fin bases of rainbow trout serve as the portal of entry for IHNV. Upon entry, the virus replicates at the fin base but not in other tissues (spleen, kidney, heart and liver) as early as 8 hours post-infection (Harmache *et al.*, 2006). In addition, fish that survived acute infection had localized bioluminescence in the fins, indicating that IHNV permissive cells are localized in the fins (Harmache *et al.*, 2006). Therefore, it is possible that in asymptomatic carrier fish, the fins may serve as a reservoir for the virus. Foott *et al.* (2006) reported that in Chinook salmon the gills and the skin support replication of the virus for almost 6 weeks in the absence of detectable virus in the internal organs. Drolet *et al.* (1995) proposed that the virus infection progresses through two parallel routes, from the mouth into the gastrointestinal tract and from the gills to the circulatory system, with systemic viraemia resulting from both routes. An ultrastructural study confirmed that the attachment and internalization of IHNV occurs via the mucosal epithelial cells of the oesophagus/cardiac/stomach region (Helmick *et al.*, 1995). Irrespective of the route of entry, typically within 24 hours post-infection a low titre of the virus is detected in gills, skin and intestine. Within 2–4 days, the virus spreads to the kidney and subsequently spreads to other organs. In young fish, the haematopoietic tissues of the kidney and spleen are severely affected (Fig. 20.2). Within 5–10 days post-exposure, young fish show signs of IHNV infection and mortalities as high as 80–100% can occur. Fish that survive IHNV infection may display spinal curvature deformities such as scoliosis and lordosis (LaPatra *et al.*, 2001).

The manifestation of IHN disease depends on the size and age of the fish, the species and strain and the environmental conditions, including water temperature (LaPatra *et al.*, 1994; LaPatra, 1998). Horizontal fish-to-fish transmission through water is the primary route through which IHNV spreads in the wild and in aquaculture operations. Although IHNV occurs primarily in fry and fingerlings in aquaculture facilities, young and older salmonids are also susceptible to the virus. There is some evidence that surviving fish can serve as lifelong carriers of the

virus. The virus titre in these carrier fish is often below the limits of serological-based detection methods. However, when these fish are immune compromised by stress associated with sexual maturation and spawning, the virus can reach a detectable level (Elston *et al.*, 1989). Increases in the density of spawning fish, eggs and fry in aquaculture operations correlates positively with IHNV outbreaks in fry (Mulachy and Bauersfeld, 1983; Foott *et al.*, 2006), perhaps due to rapid horizontal transmission of the virus.

Interactions between salmonid immune systems and IHNV have been well studied, and a number of genes involved in innate and humoral immunity have been identified (Purcell *et al.*, 2012b). IHNV infection or vaccination based on the G protein induces interferon (IFN) and IFN stimulating genes. Interestingly, IFN levels correlate with viral load but not necessarily with protection (Purcell *et al.*, 2004, 2006b, 2009; Penaranda *et al.*, 2011). This suggests that factors other than IFN are also involved in overall disease resistance. It has been proposed that the rate of viral replication perhaps competes kinetically with the expression levels of IFN (Purcell *et al.*, 2012b). The replication rate, as measured by the viral copy number, was shown to be significantly higher in an IHNV virulent strain compared with a less virulent strain and *in vivo* fitness was shown to be positively correlated with IHNV virulence (Penaranda *et al.*, 2011). Genes encoding different IFNs, including the Type I and Type II IFNs, have been identified. While the Type I IFNs were found to express in a wide range of tissues, Type II IFNs are more abundant in haematopoietic tissue (Robertsen *et al.*, 2003; Zou *et al.*, 2007; Sun *et al.*, 2008; Purcell *et al.*, 2009). Although IFNs and IFN-stimulating genes are important to initiate defence response, neutralizing antibodies are critical components of long-term adaptive immunity. Studies with attenuated or less virulent IHNV strains revealed that virus load has to attain a certain threshold before a broadly protective antibody response is developed. A comprehensive review of the antibody response in fish upon rhabdovirus infection has been published (Lorenzen and LaPatra, 1999). Recently, different immunoglobulin isotypes, IgM, IgD and IgT, have been identified in salmonids. It has been shown that IgM is expressed upon antigenic stimulation in systemic tissues, and IgT is critical for mucosal immunity (Hordvik *et al.*, 2002; Danilova *et al.*, 2005; Hansen *et al.*, 2005; Zhang *et al.*, 2010).

20.6 Disease Diagnostics

As mentioned above, the outward clinical signs of IHN can be dramatic and easily recognized. However, in early acute outbreaks, fish can die without outward signs of infection. Also, fry younger than 2 months may display few clinical signs, despite suffering a high mortality rate. In order to prevent the spread and occasional outbreaks of the disease, several methods have been developed for diagnosis and detection of IHNV (OIE, 2006). A preliminary diagnosis of the disease can be made based on fish displaying increased mortality and clinical signs of the disease that include lethargy, dark coloration of the body, distended abdomen, exopthalmia ('pop eye'), pale gills and mucoid opaque faecal casts (Fig. 20.1). A useful and quick diagnostic test for IHN is to look for 'foamy' macrophages and necrotic debris in an anterior kidney organ imprint that is stained with DiffQuik™, which is a commercially available Romanowsky stain (Fig. 20.2, panel C).

20.6.1 Virus isolation by cell culture

Isolation of the virus from suspect fish using established cell lines that is followed by confirmation using a serum neutralization assay is considered the 'gold standard' for detection of IHNV (Winton, 1991; OIE, 2006). However, cell culture for virus isolation is very time-consuming, usually taking 7–14 days to complete. More rapid identification of the virus can also be performed by immunofluorescence (IFA), enzyme-linked immunosorbent assay (ELISA), *in situ* hybridization using biotinylated probe (Deering *et al.*, 1991), immunohistochemical

and immunogold labelling (Drolet *et al.*, 1995), reverse transcriptase-PCR (RT-PCR) (Arakawa *et al.*, 1990; Chiou *et al.*, 1995; Barlic-Maganja *et al.*, 2002) and real-time RT-PCR (Overturf *et al.*, 2001; Purcell *et al.*, 2006a; Dhar *et al.*, 2008). However, virus isolation is still routinely accomplished using established fish cell lines and subsequently confirmed using many of the methods described above.

Tissue(s) to be taken from suspect fish for isolation of the virus depend(s) on the size of the fish. The preferred tissues for diagnosis are from the kidney and spleen. However, for testing adult fish, ovarian fluid is preferred because it is a non-invasive sampling methodology. Non-lethal tissue sampling using mucus swabs and pectoral fin clips has also been used for the detection and identification of the virus (LaPatra *et al.*, 1989; Dhar *et al.*, 2008). There are many fish cell lines that are susceptible to IHNV. The OIE *Manual of Diagnostic Tests for Aquatic Animals* recommends the use of *Epithelioma papulosum cyprinid* (EPC) and bluegill fry (BF-2) cell lines for IHNV isolation (OIE, 2006). The *Canadian Manual of Compliance* recommends the use of at least two of the four approved cell lines: EPC, rainbow trout gonad (RTG-2), Chinook salmon embryo (CHSE-214) or fathead minnow (FHM) cells (Canada FaO, 2004). The optimal temperature for virus growth in cell culture is 15°C, while a temperature above 23°C does not support virus replication. The use of more rapid tests for diagnostics will require more validation and approval by governmental regulators but could provide an economical and more useful tool for real-time management of IHN in the hatchery and growout facilities.

20.6.2 Serological assay

Antibody-based IHNV detection methods, such as the serum neutralization test, have been optimized and approved by OIE, as well as regulatory organizations in the USA and Canada (Canada FaO, 2004; OIE, 2006). The serum neutralization method involves incubating infectious virus (10^2–10^4 plaque forming units (pfu)/ml) with specific IHNV antibody at 15°C for 1 hour before inoculating a 24-hour-old monolayer of cells. After inoculation, the cells are incubated at 15°C and the development of any cytopathic effects is recorded. However, like virus isolation, the serum neutralization test is not rapid, taking 7–10 days before results are completed. The fluorescent antibody assay (FIA) is rapid, sensitive and specific. FIA is now accepted for detection and identification of IHNV in cell culture. Detection can be performed in 24 hours in cells showing cytopathic effect, and the limit of detection is approximately 10^3 pfu/ml.

An antigen detection assay, based on ELISA using monoclonal antibodies against the N protein or polyclonal antibodies for the detection of IHNV in cell culture, has also been approved by OIE. The assay requires less than 24 hours with a limit of detection of 10^3 pfu/ml (Ristow and Arnzen-de Avila, 1991). Although antibody-based antigen detection methods, such as FIA, ELISA and various immunohistochemical procedures, enable relatively rapid detection and identification of the virus compared with virus isolation in cell culture, various parameters, such as antibody sensitivity, specificity and sample preparation, can influence the results while negative results must be confirmed with additional testing. These techniques are not recommended for the detection of the virus in carrier fish.

20.6.3 Molecular detection

Among the different methods of IHNV detection, molecular methods are the most rapid and sensitive. However, unlike cell culture-based assays, molecular methods cannot distinguish between infectious and non-infectious virus. Reverse transcriptase-polymerase chain reaction (RT-PCR), real-time RT-PCR, molecular padlock probe (MPP) and loop-mediated isothermal amplification (LAMP)-based detection methods have been developed for the detection of IHNV. Reverse transcriptase-PCR (RT-PCR)

has been used to identify IHNV after isolating the virus in fish immortal cell lines (Arakawa *et al.*, 1990; Bruchhof *et al.*, 1995; Chiou *et al.*, 1995; Miller *et al.*, 1998; Barlic-Maganja *et al.*, 2002; Knusel *et al.*, 2007). A RT-PCR assay using primers based on the G gene is approved as a confirmatory method for IHNV isolated by cell culture (AFS-FHS, 2007). RT-PCR has been successfully used in detecting the virus in different tissue samples from IHNV-infected fish (Miller *et al.*, 1998; Barlic-Maganja *et al.*, 2002; Knusel *et al.*, 2007). Multiplex RT-PCR for simultaneous detection of IHNV, VHSV and infectious pancreatic necrosis virus (IPNV) in cell culture-derived samples has been developed (Williams *et al.*, 1999). Although RT-PCR is more sensitive than cell culture and serological methods, quantification of virus by RT-PCR is laborious, time consuming and relies on post-PCR analysis of the amplified product. These limitations have been overcome with the development of real-time PCR assays (Bustin, 2000; Niesters, 2002; Schmittgen and Livak, 2008). The real-time RT-PCR method has a greater sensitivity than conventional PCR, requires little tissue or RNA and thus could be combined with non-invasive tissue sampling methods. In addition, the method has a wide dynamic detection range, does not require post-PCR analysis and can be formatted for high throughput applications. Real-time RT-PCR using the TaqMan® probe (Roche Molecular Systems, Inc.) targeting the N and G genes was first used by Overturf *et al.* (2001) for the detection of IHNV in kidney and brain samples in trout. The sensitivity of the assay was found to be 100 copies of the viral genome per reaction. A similar assay was subsequently used to quantify the genomic (negative-sense) and replicating/transcriptionally active (positive-sense) copies of IHNV (Purcell *et al.*, 2006a). Strand-specific RT-PCR described by Purcell and colleagues is valuable for studying IHNV gene expression and pathogenesis. Recently, a multiplex real-time RT-PCR assay using the TaqMan® probe to detect and quantify IHNV and VHSV simultaneously has been published (Liu *et al.*, 2008). Since TaqMan® probes are quite expensive, a less costly assay alternative using the SYBR® Green dye (Invitrogen, Inc.) has been developed for the detection and quantification of IHNV in cell culture as well as in kidney, liver, spleen, adipose tissue and pectoral fin samples of naturally and experimentally infected rainbow trout (*O. mykiss*) (Dhar *et al.*, 2008). This study demonstrated the feasibility of SYBR® Green real-time RT-PCR using pectoral fin clips for IHNV screening in field-collected samples. In addition, since the SYBR® Green-based assay enables one to distinguish amplicons based on their melting temperature (T_m values), the IHNV detection method targeting highly variable region(s) of the viral genome flanked by conserved region for designing primers could be used to identify the emergence of novel strains of the virus. A multiplex real-time RT-PCR using TaqMan® probes (FAM, HEX and CY5) has been developed for the simultaneous detection and quantification of IHNV, VHSV and spring viraemia of carp virus (SVCV). The method was found to be specific and capable of detecting 100 copies of SVCV, 220 copies of IHNV and 140 copies of VHSV (Liu *et al.*, 2008). Such an assay has the potential to be used for rapid and large-scale screening in hatchery and breeding programmes.

A molecular padlock probe (MPP)-based assay using a single-stranded linear probe targeting an IHNV gene was described by Millard *et al.* (2006). This is an isothermal non-PCR-based nucleic acid amplification method that could successfully detect 10^4 copies of IHNV in kidney tissues of IHNV-infected rainbow trout.

Like MPP, a reverse transcription loop-mediated isothermal amplification (RT-LAMP) method was published for the detection of IHNV in visceral tissues of rainbow trout (Gunimaladevi *et al.*, 2005). The RT-LAMP method was found to be 10-fold more sensitive than nested RT-PCR. Since the LAMP method does not require a thermocycler and costly reagents, such as fluorescent probes, dyes and etc., such a method could be used as a rapid diagnostic tool for both laboratory and field applications. Although molecular methods of detection

are rapid, highly specific and more sensitive than serological detection methods, validation and standardized protocols are needed before the molecular methods can be approved by the regulatory agencies for widespread use in the aquaculture industry.

20.7 Disease Control

Control of IHN is mostly achieved by practising biosecurity and farm management. In the USA, there is no approved therapeutic for controlling IHN. The industry's focus is on careful introduction of stocks to the farm, use of virus-free water, treatment of feeds and other general methods to achieve biosecurity. However, in Canada a vaccine is available for the prevention of IHN in Atlantic salmon. This vaccine is called Apex-IHN® and is available through Novartis Animal Health and is based on technology developed by Vical (http://www.vical.com/products/infectious-disease-vaccines/Apex-IHN/default.aspx). Apex-IHN is a DNA vaccine targeting the IHNV G protein that is delivered by intramuscular injection to the fish.

Research is still ongoing to develop better methods to reduce virus release into the environment. A recent study using ultraviolet C radiation (UVC) to inactivate IHNV in fish processing wastes was able to demonstrate that the virus persisted without treatment and that treatment with UVC reduced the viral load significantly (Afonso *et al.*, 2012). A difference in the amount of radiation required for different viruses was also demonstrated in a comparison of IHNV with VHSV.

Research continues on the development of therapeutics and treatments of IHN and other fish viral diseases. For example, a recent study looked at the impact of casein and casein hydrolysates on the growth of IHNV in cell culture (Rodríguez Saint-Jean *et al.*, 2012). Additionally, prior infection of rainbow trout with IPNV conferred a strong protective effect in rainbow trout against infection with IHNV (Byrne *et al.*, 2008). The animals that were infected with IPNV had no mortality over that of the control, while those infected with IHNV alone had 72% mortality. If the IPNV-infected trout were then challenged with IHNV, only 2% of the fish died. They concluded that a protective phenomenon occurs with primary IPNV infection and this was termed interference-mediated resistance.

Oral vaccination is being investigated as a means of economical delivery of IHNV antigens and nucleic acid constructs for both salmon and rainbow trout. A recent study using nanoparticles encapsulated with poly(D,L-lactic-co-glycolic acid) (PLGA) was shown to deliver a DNA vaccine in rainbow trout (Adomako *et al.*, 2012). However, the data did not show robust expression of the DNA-delivered gene in the rainbow trout. The authors concluded that the PLGA nanoparticles were capable of delivery using an oral route, which was supported by the detection of a fluorescent dye in the gastrointestinal tract that was included in the nanoparticles. Another study demonstrated the expression of both the G and M proteins in a DNA construct where they were driven by different promoters that allowed differential control of expression (Alonso *et al.*, 2011). The apoptosis induction M gene was under the control of a metallothionein promoter that is induced on exposure to $ZnCl_2$. This would provide control as well as some biosafety for use of this vaccine. There is considerable difference between jurisdictions in the regulatory approval of nucleic acid-based vaccines and the construct tested would add an additional level of safety since it would require the presence of $ZnCl_2$ for activity. The authors also provide a nice discussion of some of the issues encountered in regulatory approval of these recombinant vaccines.

Additionally, new tools are being generated that could lead to a better understanding of IHNV as well as be applied to the production of effective vaccines. IHNV is being produced using a reverse genetics approach to test both virulence in IHNV and related viruses (e.g. VHSV) and help improve the prospects of IHNV and other fish virus vaccines (Einer-Jensen *et al.*, 2011). Prior studies have demonstrated

production of fully active IHNV in fish cell culture using vaccina virus to recover the IHNV (Biacchesi *et al*., 2000a). A recently published study developed methods for production of recombinant IHNV in fish cells using a helper virus-free method (Ammayappan *et al*., 2010). This new helper virus-free based method produced recombinant IHNV in EPC cell culture that were comparable to the parent strain, including growth characteristics and clinical manifestation in rainbow trout. This study also demonstrated that a foreign protein, in this case green fluorescent protein, could be incorporated into the virus with the DNA vaccine. These results together provide an interesting platform on which to base future production of vaccines for IHN as well as other viral diseases.

20.8 Conclusions

Since the first report of IHN in the 1950s, considerable knowledge on the prevalence of the disease, mode of disease transmission, applicable and specific diagnostic tools, and strategies for preventing negative impacts of the disease in salmonid aquaculture and in wild populations has been accumulated. Availability of a number of IHNV genome sequences has led to the development of nucleic acid-based molecular detection tools and recombinant DNA-based vaccines. Despite this significant progress, IHNV remains a major constraint in salmonid aquaculture, as exemplified by the recent outbreak of IHN in a salmon farm in the state of Washington, USA. This indicates the need for a greater emphasis on early detection and prediction of IHNV outbreaks in a manner that helps to reduce the impact of the disease. Highly sensitive IHNV detection methods have been developed, such as real-time PCR, and could be used as a signature for early detection well before an outbreak occurs. However, adoption of such ultra-sensitive methods for wide-scale application still requires an optimized and well-validated protocol provided to the industry; such a system would provide results that could be interpreted unequivocally among end users both in commercial and academic settings. These efforts are necessary before regulatory approval will be forthcoming. Currently, there is no optimized protocol for IHNV challenge experiments. As a result, comparison of data from different laboratories is difficult, since they use different IHNV isolates, viral dosages and other experimental conditions. There is also a need for further development of safe and efficacious vaccine(s) that are acceptable within the current regulatory framework. Although there is an injectable DNA vaccine against IHN commercially available in Canada, no such vaccine has been approved for commercial use in the USA. There is certainly a need for an alternative to injection vaccines. The development of an IHNV oral vaccine would fill this gap and should be a priority for future vaccine research. Toward this end, the development of an oral vaccine using a live attenuated strain(s) would probably be most desirable considering the low cost, protection efficacy and ease of administration. Developing oral vaccines is a possibility now that a reverse genetic system for IHNV has been established. The ability to genetically manipulate the IHNV genome using a reverse genetics approach has contributed to a better understanding of the role of virally encoded proteins and enabled the identification of the determinants of virulence and host-specificity. Obviously, the next several years will be quite exciting in IHNV research as the molecular tools to manipulate viral genomes are advanced, the genome sequences of salmonid fish become available and the fish immune system is better understood.

Useful Internet Resources

- An online database for IHNV in Pacific salmonid fish: http://pubs.usgs.gov/fs/2012/3027/pdf/fs20123027.pdf
- Molecular epidemiology of aquatic pathogens: http://gis.nacse.org/ihnv

- OIE IHNV Diagnostic Manual: http://www.oie.int/fileadmin/home/eng/health_standards/aahc/2010/en_chapitre_1.10.4.htm/
- The AquaPathogen X, a database for recording information on individual isolates of aquatic pathogens: http://wfrc.usgs.gov

References

Adomako, M., St-Hilaire, S., Zheng, Y., Eley, J., Marcum, R.D., Sealey, W., Donahower, B.C., Lapatra, S. and Sheridan, P.P. (2012) Oral DNA vaccination of rainbow trout, *Oncorhynchus mykiss* (Walbaum), against infectious haematopoietic necrosis virus using PLGA [poly(D,L-lactic-co-glycolic acid)] nanoparticles. *Journal of Fish Diseases* 35, 203–214.

Afonso, L., Richmond, Z., Eaves, A., Richard, J., Hawley, L. and Garver, K. (2012) Use of ultraviolet C (UVC) radiation to inactivate infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV) in fish processing plant effluent. *Journal of Aquaculture Research and Development* 3, 1–5.

AFS-FHS (2007) *Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens*. American Fisheries Society – Fish Health Section, Bethesda, Maryland.

Alonso, M., Kim, C.H., Johnson, M.C., Pressley, M. and Leong, J.A. (2004) The NV gene of snakehead rhabdovirus (SHRV) is not required for pathogenesis, and a heterologous glycoprotein can be incorporated into the SHRV envelope. *Journal of Virology* 78, 5875–5882.

Alonso, M., Chiou, P.P. and Leong, J.-A. (2011) Development of a suicidal DNA vaccine for infectious hematopoietic necrosis virus (IHNV). *Fish and Shellfish Immunology* 30, 815–823.

Amend, D.F. and Smith, L. (1975) Pathophysiology of infectious hematopoietic necrosis virus disease in rainbow trout: hematological and blood chemical changes in moribund fish. *Infection and Immunity* 11, 171–179.

Ammayappan, A., Lapatra, S.E. and Vakharia, V.N. (2010) A vaccinia-virus-free reverse genetics system for infectious hematopoietic necrosis virus. *Journal of Virological Methods* 167, 132–139.

Arakawa, C., Deering, R., Higman, K., Oshima, K., O'Hara, P. and Winton, J. (1990) Polymerase chain reaction amplification (PCR) of a nucleoprotein gene sequence of infectious haematopoietic necrosis virus. *Diseases of Aquatic Organisms* 8, 165–170.

Arkush, K.D., Mendonca, H.L., McBride, A.M. and Hedrick, R.P. (2004) Susceptibility of captive adult winter-run Chinook salmon *Oncorhynchus tshawytscha* to waterborne exposures with infectious hematopoietic necrosis virus (IHNV). *Diseases of Aquatic Organisms* 59, 211–216.

Banerjee, A.K. and Barik, S. (1992) Gene expression of vesicular stomatitis virus genome RNA. *Virology* 188, 417–428.

Barlic-Maganja, D., Strancar, M., Hostnik, P., Jencic, V. and Grom, J. (2002) Comparison of the efficiency and sensitivity of virus isolation and molecular methods for routine diagnosis of infectious haematopoietic necrosis virus and infectious pancreatic necrosis virus. *Journal of Fish Diseases* 25, 73–80.

Bergmann, S.M., Fichtner, D., Skall, H.F., Schlotfeldt, H.J. and Olesen, N.J. (2003) Age- and weight-dependent susceptibility of rainbow trout *Oncorhynchus mykiss* to isolates of infectious haematopoietic necrosis virus (IHNV) of varying virulence. *Diseases of Aquatic Organisms* 55, 205–210.

Biacchesi, S. (2011) The reverse genetics applied to fish RNA viruses. *Veterinary Research* 42, 12.

Biacchesi, S., Thoulouze, M.I., Bearzotti, M., Yu, Y.X. and Bremont, M. (2000a) Recovery of NV knockout infectious hematopoietic necrosis virus expressing foreign genes. *Journal of Virology* 74, 11,247–11,253.

Biacchesi, S., Yu, Y.X., Bearzotti, M., Tafalla, C., Fernandez-Alonso, M. and Bremont, M. (2000b) Rescue of synthetic salmonid rhabdovirus minigenomes. *Journal of General Virology* 81, 1941–1945.

Bootland, L. and Leong, J.-A.C. (2011) Infectious haematopoietic necrosis virus. In: Woo, P. and Brunol, D. (eds) *Fish Diseases and Disorders*, vol. 3. CAB International, Wallingford, UK, pp. 66–109.

Bourhy, H., Cowley, J.A., Larrous, F., Holmes, E.C. and Walker, P.J. (2005) Phylogenetic relationships among rhabdoviruses inferred using the L polymerase gene. *Journal of General Virology* 86, 2849–2858.

Bruchhof, B., Marquardt, O. and Enzmann, P.J. (1995) Differential diagnosis of fish pathogenic rhabdoviruses by reverse transcriptase-dependent polymerase chain reaction. *Journal of Virological Methods* 55, 111–119.

Brudeseth, B.E., Castric, J. and Evensen, O. (2002) Studies on pathogenesis following single and double infection with viral hemorrhagic septicemia virus and infectious hematopoietic necrosis virus in rainbow trout (*Oncorhynchus mykiss*). *Veterinary Pathology* 39, 180–189.

Bustin, S.A. (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *Journal of Molecular Endocrinology* 25, 169–193.

Byrne, N., Castric, J., Lamour, F., Cabon, J. and Quentel, C. (2008) Study of the viral interference between infectious pancreatic necrosis virus (IPNV) and infectious haematopoietic necrosis virus (IHNV) in rainbow trout (*Oncorhynchus mykiss*). *Fish & Shellfish Immunology* 24, 489–497.

Canada FaO (2004) *Fish Health Protection Regulations: Manual of Complicance (Revised)*. Fisheries and Oceans Canada, Ottawa.

Chiou, P., Droler, B. and Leong, J. (1995) Polymerase chain reaction amplification of infectious haematopoietic necrosis virus RNA extracted from fixed and embedded fish tissue. *Journal of Aquatic Animal Health* 7, 9–15.

Chiou, P.P., Kim, C.H., Ormonde, P. and Leong, J.A. (2000) Infectious hematopoietic necrosis virus matrix protein inhibits host-directed gene expression and induces morphological changes of apoptosis in cell cultures. *Journal of Virology* 74, 7619–7627.

Choi, M.K., Moon, C.H., Ko, M.S., Lee, U.H., Cho, W.J., Cha, S.J., Do, J.W., Heo, G.J., Jeong, S.G., Hahm, Y.S., Harmache, A., Bremont, M., Kurath, G. and Park, J.W. (2011) A nuclear localization of the infectious haematopoietic necrosis virus NV protein is necessary for optimal viral growth. *PLoS One* 6, e22362.

Congleton, J.L. (1988) Getting in control of deadly IHN. *Focus Renew Research* 14, 18–19.

Danilova, N., Bussmann, J., Jekosch, K. and Steiner, L.A. (2005) The immunoglobulin heavy-chain locus in zebrafish: identification and expression of a previously unknown isotype, immunoglobulin Z. *Nature Immunology* 6, 295–302.

Deering, R.M, Arakawa, C.K., Oshima, K.H., O'Hara, P.J., Landolt, M.L. and Winton, J.R. (1991) Development of a biotinylated DNA probe for detection and identification of infectious haematopoietic necrosis virus. *Disease of Aquatic Organisms* 11, 57–65.

Dhar, A.K., Bowers, R.M., Licon, K.S. and Lapatra, S.E. (2008) Detection and quantification of infectious hematopoietic necrosis virus in rainbow trout (*Oncorhynchus mykiss*) by SYBR Green real-time reverse transcriptase-polymerase chain reaction. *Journal of Virological Methods* 147, 157–166.

Drolet, B.S., Chiou, P.P., Heidel, J. and Leong, J.A. (1995) Detection of truncated virus particles in a persistent RNA virus infection in vivo. *Journal of Virology* 69, 2140–2147.

Einer-Jensen, K., Biacchesi, S., Stegmann, A., Bremont, M. and Lorenzen, H. (2011) Recombinant hybrid infectious hematopoietic necrosis virus (IHNV) carrying viral haemorrhagic septicaemia virus (VHSV) G or Nv genes show different virulence properties. *15th International Conference on Diseases of Fish and Shellfish*, Split, Croatia.

Elston, R.A., Harrell, L.W. and Flagg, T.A. (1989) How do adult sockeye salmon contact IHNV disease? *Fish Health Section, American Fisheries Society Newsletter* 17, 4.

Engelking, H.M. and Leong, J.C. (1989) The glycoprotein of infectious hematopoietic necrosis virus elicits neutralizing antibody and protective responses. *Virus Research* 13, 213–230.

Foott, J., Free, D., McDowell, T., Arkush, K. and Hedrick, R. (2006) Infectious hematopoietic necrosis virus transmission and disease among juvenile chinook salmon exposed in culture compared to environmentally relevant conditions. *San Francisco Estuary Watershed Science* 4, 1–16.

Garver, K.A., Troyer, R.M. and Kurath, G. (2003) Two distinct phylogenetic clades of infectious hematopoietic necrosis virus overlap within the Columbia River basin. *Diseases of Aquatic Organisms* 55, 187–203.

Gilmore, R.D., Jr and Leong, J.A. (1988) The nucleocapsid gene of infectious hematopoietic necrosis virus, a fish rhabdovirus. *Virology* 167, 644–648.

Gunimaladevi, I., Kono, T., Lapatra, S.E. and Sakai, M. (2005) A loop mediated isothermal amplification (LAMP) method for detection of infectious hematopoietic necrosis virus (IHNV) in rainbow trout (*Oncorhynchus mykiss*). *Archives of Virology* 150, 899–909.

Hansen, J.D., Landis, E.D. and Phillips, R.B. (2005) Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: implications for a distinctive B cell developmental pathway in teleost fish. *Proceedings of the National Academy of Sciences USA* 102, 6919–6924.

Harmache, A., LeBerre, M., Droineau, S., Giovannini, M. and Bremont, M. (2006) Bioluminescence imaging of live infected salmonids reveals that the fin bases are the major portal of entry for Novirhabdovirus. *Journal of Virology* 80, 3655–3659.

Helmick, C., Bailey, F., LaPatra, S. and Ristow, S. (1995) The esophagus/cardiac stomach region: site of attachment and internalization of infectious hematopoieetic necrosis virus in challenged juvenile rainbow trout (*Oncorhynchus mykiss*) and coho salmon (*L. kisutch*). *Diseases of Aquatic Organisms* 23, 189–199.

Hordvik, I., Berven, F.S., Solem, S.T., Hatten, F. and Endresen, C. (2002) Analysis of two IgM isotypes in Atlantic salmon and brown trout. *Molecular Immunology* 39, 313–321.

Jeon, C.-H., Suebssing, R., Hong, K.-E., Oh, M.-J. and Kim, J.-H. (2012) Monitoring fish pathogenic viruses from chum salmon (*Oncorhynchus keta*) in Korea. *North Pacific Anadromous Fish Commission Bulletin* 4, 155–157.

Knusel, R., Bergmann, S.M., Einer-Jensen, K., Casey, J., Segner, H. and Wahli, T. (2007) Virus isolation vs RT-PCR: which method is more successful in detecting VHSV and IHNV in fish tissue sampled under field conditions? *Journal of Fish Diseases* 30, 559–568.

Kurath, G. and Leong, J.C. (1985) Characterization of infectious hematopoietic necrosis virus mRNA species reveals a nonvirion rhabdovirus protein. *Journal of Virology* 53, 462–468.

Kurath, G., Higman, K.H. and Bjorklund, H.V. (1997) Distribution and variation of NV genes in fish rhabdoviruses. *Journal of General Virology* 78, 113–117.

Kurath, G., Garver, K.A., Troyer, R.M., Emmenegger, E.J., Einer-Jensen, K. and Anderson, E.D. (2003) Phylogeography of infectious haematopoietic necrosis virus in North America. *Journal of General Virology* 84, 803–814.

Kurath, G., Jolley, J., Thompson, T., Thompson, D., Whitesel, T., Gutenberger, S. and Winton, J. (2012) Larval Pacific lamprey *Entosphenus tridentatus* are not susceptible to common fish rhabdoviruses of the Pacific Northwest. *142nd Annual Meeting American Fisheries Society*, Minneapolis, Minnesota.

LaPatra, S.E. (1998) Factors affecting pathogenecity of infectious hematopoietic necrosis virus (IHNV) for salmonid fish. *Journal of Aquatic Animal Health* 10, 121–131.

LaPatra, S., Rohovex, J. and Fryer, J. (1989) Detection of infectious hematopoietic necrosis virus in fish mucus. *Fish Pathology* 14, 197–202.

LaPatra, S., Groff, J.M., Fryer, J.L. and Hedrick, R.P. (1990) Comparative pathogenesis of three strains of infectious haematopoietic necrosis virus in rainbow trout (*Oncorhynchus mykiss*). *Diseases of Aquatic Organisms* 8, 105–112.

LaPatra, S.E., Lauda, K.A. and Jones, G.R. (1994) Antigenic variants of infectious haematopoietic necrosis virus and implications for vaccine development. *Diseases of Aquatic Organisms* 20, 119–126.

LaPatra, S.E., Batts, W.N., Overturf, K., Jones, G.R., Shewmaker, W.D. and Winton, J.R. (2001) A risk associated with the movement of processed rainbow trout, *Oncorhynchus mykiss*, from an infectious hematopoietic necrosis virus (IHNV) endemic area. *Journal of Fish Diseases*, 399–408.

Leong, J. and Kurath, G. (2010) Novirhabdoviruses, Rhabdoviridae. In: Tidona, C. and Darai, G. (eds) *Springer Index of Viruses*. Springer, Heidelberg, Germany.

Liu, Z., Teng, Y., Liu, H., Jiang, Y., Xie, X., Li, H., Lv, J., Gao, L., He, J., Shi, X., Tian, F., Yang, J. and Xie, C. (2008) Simultaneous detection of three fish rhabdoviruses using multiplex real-time quantitative RT-PCR assay. *Journal of Virological Methods* 149, 103–109.

Lorenzen, N. and LaPatra, S.E. (1999) Immunity to rhabdoviruses in rainbow trout: the antibody response. *Fish and Shellfish Immunology* 9, 345–360.

MacKinnon, A.M., Cook, T., Hawley, L.L., Traxler, G., Morrison, D. and Salonius, K. (2008) Efficient IHN vaccine in Atlantic salmon subjected to an IHNV challenge simulating natural and/or e challenges. *American Fisheries Society – Fish Health section, Annual Meeting*.

McAllister, P.E. and Wagner, R.R. (1975) Structural proteins of two salmonid rhabdoviruses. *Journal of Virology* 15, 733–738.

Millard, P., Bickerstaff, L., LaPatra, S. and Kim, C. (2006) Detection of infectious hematopoietic necrosis virus and infectious salmon anemia virus by molecular padlock amplification. *Journal of Fish Diseases* 29, 201–213.

Miller, T.A., Rapp, J., Wastlhuber, U., Hoffmann, R.W. and Enzmann, P.J. (1998) Rapid and sensitive reverse transcriptase-polymerase chain reaction based detection and differential diagnosis of fish pathogenic rhabdoviruses in organ samples and cultured cells. *Diseases of Aquatic Organisms* 34, 13–20.

Morzunov, S.P., Winton, J.R. and Nichol, S.T. (1995) The complete genome structure and phylogenetic relationship of infectious hematopoietic necrosis virus. *Virus Research* 38, 175–192.

Mulcahy, D., Burke, J., Pascho, R. and Jenes, C. (1982) Pathogenesis of infectious hematopoietic necrosis virus in spawning sockeye salmon (*Oncorhynchus nerka*). *Canadian Journal of Fish Aquatic Sciences* 39, 1144–1149.

Mulachy, D. and Bauersfeld, K. (1983) Effect of loading density of sockeye salmon, *Oncorhychus nerka* (Walbaum), eggs in incubation boxes on mortality caused by infectious haematopoietic necrosis virus. *Journal of Fish Diseases* 8, 383–396.

Niesters, H.G. (2002) Clinical virology in real time. *Journal of Clinical Virology* 25, S3–12.

OIE (2006) *Manual of Diagnostic Tests for Aquatic Animal Diseases*. Office International des Epizootics, Paris.

Overturf, K., LaPatra, S. and Powell, M. (2001) Real-time PCR for the detection and quantitative analysis of IHNV in salmonids. *Journal of Fish Diseases* 24, 325–333.

Penaranda, M.M.D., Wargo, A.R. and Kurath, G. (2011) In vivo fitness correlates with host-specific virulence of infectious haematopoietic necrosis virus (IHNV) in sockeye salmon and rainbow trout. *Virology* 417, 312–319.

Poch, O., Blumberg, B.M., Bougueleret, L. and Tordo, N. (1990) Sequence comparison of five polymerases (L proteins) of unsegmented negative-strand RNA viruses: theoretical assignment of functional domains. *Journal of General Virology* 71, 1153–1162.

Purcell, M.K., Kurath, G., Garver, K.A., Herwig, R.P. and Winton, J.R. (2004) Quantitative expression profiling of immune response genes in rainbow trout following infectious haematopoietic necrosis virus (IHNV) infection or DNA vaccination. *Fish and Shellfish Immunology* 17, 447–462.

Purcell, M.K., Hart, S.A., Kurath, G. and Winton, J.R. (2006a) Strand-specific, real-time RT-PCR assays for quantification of genomic and positive-sense RNAs of the fish rhabdovirus, infectious hematopoietic necrosis virus. *Journal of Virological Methods* 132, 18–24.

Purcell, M.K., Nichols, K.M., Winton, J.R., Kurath, G., Thorgaard, G.H., Wheeler, P., Hansen, J.D., Herwig, R.P. and Park, L.K. (2006b) Comprehensive gene expression profiling following DNA vaccination of rainbow trout against infectious hematopoietic necrosis virus. *Molecular Immunology* 43, 2089–2106.

Purcell, M.K., Laing, K.J., Woodson, J., Thorgaard, G. and Hansen, J.D. (2009) Characterization of the interferon genes in homozygous rainbow trout reveals two novel genes, alternate splicing and differential regulation of duplicated genes. *Fish and Shellfish Immunology* 26, 293–304.

Purcell, M., Lapatra, S., Woodson, J., Kuratah, G. and Winton, J. (2012a) Early viral replication and induced or constitutive immunity in rainbow trout familes with differential resistance to infectious hematopoietic necrosis virsus (IHNV). *Fish and Shellfish Immunology* 28, 98–105.

Purcell, M.K., Laing, K.J. and Winton, J.R. (2012b) Immunity to fish rhabdoviruses. *Viruses* 4, 140–166.

Ristow, S. and Arnzen-de Avila, J. (1991) Monoclonal antibodies to the glycoprotein and nucleoprotein of infectious hematopoietic necrosis virus (IHNV) reveal differences among isolates of the virus by fluorescence, neutralization and electrophoresis. *Diseases of Aquatic Organisms* 11, 105–115.

Robertsen, B., Bergan, V., Rokenes, T., Larsen, R. and Albuquerque, A. (2003) Atlantic salmon interferon genes: cloning, sequence analysis, expression, and biological activity. *Journal of Interferon and Cytokine Research* 10, 601–612.

Rodríguez Saint-Jean, S., De las Heras, A., Carrillo, W., Recio, I., Ortiz-Delgado, J.B., Ramos, M., Gomez-Ruiz, J.A., Sarasquete, C. and Pérez-Prieto, S.I. (2012) Antiviral activity of casein and αs2 casein hydrolysates against the infectious haematopoietic necrosis virus, a rhabdovirus from salmonid fish. *Journal of Fish Diseases* 36, 467–481.

Schmittgen, T.D. and Livak, K.J. (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nature Protocole* 3, 1101–1108.

Schutze, H., Enzmann, P.J., Kuchling, R., Mundt, E., Niemann, H. and Mettenleiter, T.C. (1995) Complete genomic sequence of the fish rhabdovirus infectious haematopoietic necrosis virus. *Journal of General Virology* 76, 2519–2527.

Sun, B., Robertsen, B., Wang, Z. and Bin, L. (2008) Identification of an Atlantic salmon IFN multigene cluster encoding three IFN subtypes with very different expression properties. *Developmental and Comparative Immunology* 33, 547–558.

Thoulouze, M.I., Bouguyon, E., Carpentier, C. and Bremont, M. (2004) Essential role of the NV protein of Novirhabdovirus for pathogenicity in rainbow trout. *Journal of Virology* 78, 4098–4107.

Troyer, R.M., LaPatra, S.E. and Kurath, G. (2000) Genetic analyses reveal unusually high diversity of infectious haematopoietic necrosis virus in rainbow trout aquaculture. *Journal of General Virology* 81, 2823–2832.

van Regenmortel, M., Fauquet, C., Bishop, D., Carstens, E., Estes, M., Lemon, S., Maniloff, J., Mayo, M., McGeoch, D., Pringle, C. and Genus, R. (2000) *Novirhabdovirus. Seventh Report on the International Committee on Taxonomy of Viruses*. Academic Press, New York.

Walker, P.J. and Winton, J.R. (2010) Emerging viral diseases of fish and shrimp. *Veterinary Research* 41, 51.

Williams, K., Blake, S., Sweeney, A., Singer, J.T. and Nicholson, B.L. (1999) Multiplex reverse transcriptase PCR assay for simultaneous detection of three fish viruses. *Journal of Clinical Microbiology* 37, 4139–4141.

Winton, J. (1991) Recent advances in the detection and control of infectious haematopoietic necrosis virus. *Annual Review in Fish Diseases* 2, 83–93.

Yamamoto, T., Batts, W., Arakawa, C. and Winton, J. (1990) Multiplication of infectious hematopoietic necrosis virus in rainbow trout following immersion infection: whole-body assay and immunohistochemistry. *Journal of Aquatic Animal Health* 2, 271–280.

Zhang, Y.A., Salinas, I., Li, J., Parra, D., Bjork, S., Xu, Z., LaPatra, S.E., Bartholomew, J. and Sunyer, J.O. (2010) IgT, a primitive immunoglobulin class specialized in mucosal immunity. *Nature Immunology* 11, 827–835.

Zou, J., Tafalla, C., Truckle, J. and Secombes, C.J. (2007) Identification of a second group of type I IFNs in fish sheds light on IFN evolution in vertebrates. *Journal of Immunology* 179, 3859–3871.

21 Viral Haemorrhagic Septicaemia Virus

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21.1 Introduction

Rhabdoviruses are important pathogens of both wild and farmed fish throughout the Northern hemisphere. The rhabdoviruses affecting fish are assigned to the two genera, *Novirhabdovirus* and *Vesiculovirus*. Viral haemorrhagic septicaemia virus (VHSV) and infectious haematopoietic necrosis virus (IHNV) are the two most important viruses in the *Novirhabdovirus* genus (Dietzgen *et al*., 2012), which also include other fish viruses such as hirame rhabdovirus and snakehead virus. In the *Vesiculovirus* genus, the most severe viruses for fish are spring viraemia of carp virus (SVCV) (Dietzgen *et al*., 2012) and the perch rhabdoviruses (tentatively assigned to the genus) (Stone *et al*., 2011).

VHSV is enveloped and contains a single-stranded, negative-sense RNA genome of 11,100 nucleotides encoding five structural and one non-structural protein. VHSV can be split into four distinct genotypes and seven subtypes, with varying geographical distribution, host range and pathogenicity. Serologically the virus is, however, very homogeneous and all isolates can be identified by immunochemical test using the monoclonal antibody (MAb) IP5B11 (Lorenzen *et al*., 1988). For decades, viral haemorrhagic septicaemia (VHS) was regarded as the most important viral disease of rainbow trout only in European aquaculture, but from the 1990s the virus was also reported to cause significant mortality and morbidity in wild fish, especially on the Pacific West coast of North America and in the Great Lakes region of the USA and Canada, causing severe die-offs. The disease is reportable to the World Organization for Animal Health (OIE).

Infected fish usually display non-specific clinical signs in the early stages of infection, including rapid onset of mortality (which can reach up to 100% in fry), lethargy, darkening of the skin, exophthalmia, anaemia (pale gills), haemorrhages at the base of the fins, gills, eyes and skin and a distended abdomen due to oedema in the peritoneal cavity. In the chronic state of infection, affected fish do not in general exhibit external signs. VHS can also occur in a nervous form, characterized by severe abnormal swimming behaviour, such as constant flashing and/or spiralling. In aquaculture the diseases have for more than 50 years been controlled by preventative measures and by eradication procedures when introduced. Vaccines have been developed but are not commercialized yet.

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21.2 Description of VHSV

VHSV is member of the genus of *Novirhabdovirus* (Dietzgen *et al.*, 2012), which consists only of fish viruses (infectious haematopoietic necrosis virus (IHNV) (type strain virus), VHSV, hirame rhabdovirus (HIRRV), snakehead virus (SHRV) and a couple of eel viruses (B12 and C26), the latter only tentatively assigned to the genus). The virion is enveloped and contains a single-stranded negative-sense RNA genome of 11,100 nucleotides. The six genes encode the five structural proteins: nucleocapsid- (N), phospho- (P), matrix- (M), glyco- (G) and RNA dependent RNA polymerase (L) protein and the non-structural protein NV (non-virion, giving name to the genus) (Schütze *et al.*, 1999). Additionally, VHSV possesses the non-coding 3′ leader and 5′ trailer sequences, like all other rhabdoviruses.

VHSV enters the cell by receptor-mediated endocytosis, most likely by binding of the viral G-protein to a fibronectin complex (Bearzotti *et al.*, 1999) and thereby fusing with the cell membrane. The viral genes are transcribed in the cytoplasm by the RNA polymerase included in the virion. The viral proteins and the full length genome are synthesized by the host-cell machinery. The RNA genome is packed with the N, P and L proteins to form the ribonucleoprotein (RNP) core, which is then associated with M to produce the RNP-M complex. In the meantime, the G protein is synthesized in the endoplasmic reticulum (ER) system where it is glycosylated and further modified in the Golgi apparatus and transported to and inserted in the plasma membrane. Here the RNP-M complex will capture it and the new virion will leave the cell by budding as a full enveloped virus (Purcell *et al.*, 2012). The NV protein is expressed at variable levels in infected cells but is not detectable in purified virions.

21.2.1 Genotypes

VHSV isolates can be divided into four major genotypes (I, II, III and IV) and seven subtypes (Ia–Ie and IVa–IVb), with an almost distinct geographical distribution (Einer-Jensen *et al.*, 2004; Elsayed *et al.*, 2006). Furthermore, there are a number of unclassified isolates of older dates within genotype I. The host range and the pathogenicity appear, at least to some extent, to be linked to the genotype of VHSV. The genotypes are identified, based on sequencing of full-length and/or truncated genes from the N-gene (Snow *et al.*, 1999, 2004; Einer-Jensen *et al.*, 2005a; Elsayed *et al.*, 2006; Gagné *et al.*, 2007), and the G-gene (Nishizawa *et al.*, 2002, 2006; Einer-Jensen *et al.*, 2004, 2005a, 2005b; Elsayed *et al.*, 2006; Gagné *et al.*, 2007; Gadd *et al.*, 2010, 2011), whereas the full-length G-gene sequencing provides the most comprehensive subdivision of VHSV (Einer-Jensen *et al.*, 2005a). The following description of genotypes is based on the full-length G-gene.

Genotype Ia: Almost all VHSV isolates causing outbreaks in European rainbow trout farms cluster in sub-lineage Ia, of which isolates have been reported from most continental European countries (Einer-Jensen *et al.*, 2004; Snow *et al.*, 2004; Toplak *et al.*, 2010; Kahns *et al.*, 2012). However, genotype Ia isolates have also been detected in species such as brown trout (*Salmo trutta*), pike (*Esox lucius*) and grayling (*Thymallus thymallus*) (de Kinkelin and Le Berre, 1977; Jonstrup *et al.*, 2009). Most genotype Ia isolates have caused outbreaks in freshwater farms, but isolates have also been obtained from rainbow trout in seawater net pens and in at least one case from turbot (Schlotfeldt *et al.*, 1991; Snow *et al.*, 2004). Genotype Ia can be further subdivided into two major subpopulations, Ia-1 and Ia-2, likewise with a distinct geographic distribution (Kahns *et al.*, 2012).

Genotype Ib: Viruses of this genotype have been isolated from fish in the marine environment in the Baltic Sea, Kattegat, Skagerrak, the North Sea and the English Channel (Einer-Jensen *et al.*, 2004; Snow *et al.*, 2004; Skall *et al.*, 2005) and in one case in Japan (Nishizawa *et al.*, 2002). None of the isolations from wild fish have been associated with clinical disease outbreaks. Genotype Ib

have only in two cases been associated with evidence of transfer between wild fish and farmed rainbow trout, in pen-reared rainbow trout close to Gothenburg, Sweden, in 1998 and 2000 (Nordblom, 1998; Nordblom and Norell, 2000; Skall *et al.*, 2005).

Genotype Ic: It is a smaller group consisting of Danish farmed rainbow trout isolates from earlier dates. Isolates of this genotype have also been identified in Germany and Austria (Jonstrup *et al.*, 2009).

Genotype Id: This group consists of some old Scandinavian isolates from the 1960s until the first VHS outbreaks occurred in Finland in sea-reared rainbow trout in 2000 at two different areas, where all isolates sampled proved to cluster in the Id genotype. In infection trials it was demonstrated that the isolates were pathogenic to rainbow trout, but less virulent than most Ia isolates (Raja-Halli *et al.*, 2006).

Genotype Ie: These isolates have been obtained from both freshwater and marine (the Black Sea) environments in Georgia and Turkey. Isolations were from both farmed and wild turbot (*Psetta maxima*) (Kalayci *et al.*, 2006; Nishizawa *et al.*, 2006; Jonstrup *et al.*, 2009) and from rainbow trout (Einer-Jensen *et al.*, 2004). VHSV has also been identified in whiting (*Merlangius merlangus*) from the Black Sea, but these isolates have not been sequenced so far (Altuntas and Ogut, 2010). However, it is very likely that they belong to this genotype.

Genotype II: The members of this group consists of marine isolates from wild fish in the Baltic Sea, including the Gulf of Bothnia and the Gulf of Finland, especially in Atlantic herring (*Clupea harengus*) (Snow *et al.*, 2004; Gadd *et al.*, 2011) and in lamprey (*Lampetra fluviatilis*) from the rivers Kalajoki and Lestijoki having outlet into the Golf of Bothnia (Gadd *et al.*, 2010).

Genotype III: These isolates originate from wild and farmed fish in the North Atlantic Sea from the Flemish Cap (López-Vázquez *et al.*, 2006) to the Norwegian coast (Dale *et al.*, 2009), the North Sea, around the British Isles, Skagerrak and Kattegat. VHS outbreaks in farmed turbot in the UK and Ireland in the 1990s were due to genotype III isolates and in 2007 an outbreak in sea-reared rainbow trout on the Norwegian West coast was due to the same genotype.

Genotype IV: All isolates originate, in this group, from North America and Asia (Skall *et al.*, 2005); however, these are divided into the two sublineages IVa and IVb. The IVa is primarily restricted to the marine environment in both North America and Asia (Meyers and Winton, 1995; Nishizawa *et al.*, 2002; Hedrick *et al.*, 2003; Kim *et al.*, 2003; Lee *et al.*, 2007; Winton *et al.*, 2008), whereas sublineage IVb isolates have been observed in both freshwater (the Great Lakes) and marine systems on the east coast of North America only (Gagné *et al.*, 2007; Winton *et al.*, 2008; Thompson *et al.*, 2011; Faisal *et al.*, 2012), where in the past decade it has caused severe die-offs in the Great Lakes region. In North America, VHSV genotype IVa has likewise caused severe epidemics in Pacific herring (*Clupea pallasii*) and to a lower extent in Atlantic salmon (*Salmo salar*) on the Pacific coast (Skall *et al.*, 2005). It has been suggested that the VHSV isolates from the North American east coast belong to a separate subtype IVc (Pierce and Stepien, 2012a, 2012b).

21.2.2 Serotypes

Serologically VHSV has been shown to be rather homogeneous and most polyclonal antibodies raised in rabbits against VHSV cross react with all known VHSV isolates in immunochemical tests such as immunofluorescence antibody technique (IFAT) and enzyme-linked immunosorbent assay (ELISA). One MAb, IP5B11, has proven to react specifically with all known existing VHSV isolates (more than 2000 isolates tested worldwide), and a positive reaction with this MAb is included as a criteria for VHSV identification in the OIE standard (OIE, 2009). In neutralization tests, however, VHSV can be divided serologically into three subgroups based on the neutralization pattern towards a panel of four neutralizing MAbs and one polyclonal antibody (Olesen *et al.*, 1993). VHSV thus shares several antigenic epitopes, although the sero-grouping does not correlate with

genotypes. MAbs specifically reacting with different genogroups of VHSV, e.g. IVa, IVb, Ib and III, have been developed (Ito *et al.*, 2010, 2011) and it is today possible to discriminate between the four genotypes and five subtypes of VHSV using a panel of nine MAbs in ELISA or by IFAT. Interestingly, a few isolates of specific genotypes did not react with MAbs in the expected manner. These isolates also differed in pathogenicity towards rainbow trout, pointing at viral epitopes that may be involved in pathogenicity. In fish specific antibodies are raised following immunization and infection.

21.3 The Disease

VHS causes disease in a large variety of fish species, and a link between genotype and pathogenicity is especially observed for rainbow trout, where the vast majority of outbreaks are caused by genotype Ia or other genotype I subtypes. Infection trials have shown that rainbow trout are relatively less susceptible for VHSV isolates of other genotypes (Skall *et al.*, 2004, 2005), although an outbreak caused by genotype III has been reported (Dale *et al.*, 2009). For other fish species, the link between pathogenicity and genotype is not as strong as for rainbow trout.

The disease in farmed rainbow trout is characterized by haemorrhages as a result of destruction of endothelial cells with bleeding in the meninges, serous surfaces, muscles, internal organs and in the eyes, and with pale gills due to anaemia. Exophthalmia and darkening of the body are consistent findings as well. Ascites can occasionally be observed (Wolf, 1988). Mortality depends on the age of the fish, which can be up to 100% in fry, often less in older fish, typically from 15% to 70%. No clinical signs are pathognomonic for VHS, but cases with dark lethargic fish with exophthalmia that do not try to escape when handling should be regarded as a VHS suspicion (Fig. 21.1). At necropsy, pale gills are seen together with empty, yellowish atonic back gut, distinct bleedings on serous surfaces and especially petechial bleeding in the dorsal musculature (Fig. 21.2).

Fig. 21.1. Rainbow trout suffering from VHS. The fish is dark, has exophthalmia and does not try to escape the net.

Fig. 21.2. Rainbow trout suffering from VHS. Notice the petechial bleeding on the intestines, the empty, yellowish atonic back gut, and the swollen kidney and spleen.

The VHSV reservoirs are clinically infected fish and covert carriers among cultured, feral or wild fish and the carrier status of VHSV in freshwater fish species is well established (Jørgensen, 1982; Enzmann and Konrad, 1985). Virus is shed with urine and ovarian fluids, whereas kidney, heart and spleen are the sites in which virus is most abundant (Wolf, 1988) and are also the target organs in rainbow trout (Brudeseth *et al.*, 2002), where especially endothelial cells lining the blood vessels are affected. Once VHSV is established in a farmed stock and, therefore, in a water catchment system, the disease becomes enzootic because of latent virus carrier fish.

The first records of a disease with symptoms similar to VHS date back to the 1930s (Schäperclaus, 1938), but the viral aetiology was not confirmed until 1962 when Jensen (1963) made the first virus isolation on trout cell cultures, and proposed the name Egtved virus, named after the Danish village from where the disease was first observed in Denmark (Jensen, 1965).

The first VHSV isolation from wild fish in the marine environment dates back to 1979 and was from Atlantic cod (*Gadus morhua*) caught in the coastal waters in the southern part of Denmark (Jensen *et al.*, 1979; Vestergård Jørgensen and Olesen, 1987), from pacific cod (*Gadus macrocephalus*) in 1990 (Meyers and Winton, 1995) and from haddock (*Melanogrammus aeglefinus*) and cod caught in Scottish east coast waters in 1993 and 1995 (Smail, 1995, 2000).

VHSV was not found outside Europe until 1988, when it was detected in ovarian fluid from ascending chinook (*Oncorhynchus tshawytscha*) and coho salmon (*O. kisutch*) in the USA (Hopper, 1989; Brunson *et al.*, 1989).

VHS is widely distributed in the continental part of Europe, having intensive production of rainbow trout in freshwater aquaculture. By August 2013 the whole coastal and continental area of the UK, Ireland, Sweden, Iceland and Norway, inland Finland and Denmark, and distinct areas and compartments in France, Germany and Spain had been approved as VHS-free zones. In these zones there are extensive programmes of monitoring farmed freshwater and marine fish. In Spain, the virus has not officially been diagnosed since 1996

(OIE International Database on Aquatic Animal Diseases (IDAAD), http://www.cefas.defra.gov.uk/idaad/) and it has never been diagnosed in Portugal or in Greece (Olesen, 1998; Ariel and Olesen, 2002; IDAAD (http://www.cefas.defra.gov.uk/idaad/)).

The widespread occurrence of VHSV in wild marine fish species in northern Europe raises concern on coastal zone status for VHS, and the risk of contracting VHS in inland waters due to diadromous fish migrating upstream into fresh waters. Based on many years of practical experience, it is however considered that the risk of contracting VHS in fresh water is very low as long as proper biosecurity measures are followed, such as not feeding with fresh fish, and no transfer of live fish from sea to fresh water. For susceptible fish reared in sea water the risk is higher, but also in this situation risk can be reduced by proper management procedures such as preventing fish wintering in sea water at very low temperatures, preventing stocking with small fish not properly habituated to salt water, or feeding with raw fish, as was the case with two outbreaks in turbot in Scotland and Ireland, respectively (Skall *et al.*, 2005).

In North America the disease has primarily been a threat to wild and feral fish, while VHS has never occurred in farmed rainbow trout on this continent.

21.3.1 Host species

During the past two decades VHSV has been isolated from an increasing number of marine and freshwater fish species. It is likely that VHSV is endemic in fish populations in large areas of the temperate northern hemisphere. So far, VHSV has been isolated from approximately 80 different fish species throughout North America, Asia and Europe. A number of species have been shown to be susceptible to VHSV under experimental conditions. The number of possible host species is increasing with increasing monitoring efforts. The most susceptible farmed fish species is rainbow trout to VHSV genotype Ia, although VHSV genotypes Ie, III and IVa also cause mortality in farmed turbot and Japanese flounder, respectively (Skall *et al.*, 2005). Among wild fish, severe die-offs have been observed in recent years in the Great Lakes region of the USA and Canada involving at least 28 freshwater fish species (USDA-APHIS-VS, 2008). All VHSV isolates from these outbreaks belong to genotype IVb.

21.3.2 Susceptible stages of the host

Infection with VHSV may cause disease and mortality in all life stages of susceptible fish. Infection may result in the development of protective immunity in endemic areas; the disease is therefore more abundant in populations of young, not previously infected fish. VHSV is not known to infect fish eggs. In Pacific herring, high mortalities were observed in mature fish returning for spawning along the coast and experimental infection trials revealed high mortalities in early stages of their life cycles (Kocan *et al.*, 1997). In Europe, however, isolation of VHSV was not connected with obvious clinical signs in the fish. Only mature herring (≥1 year) are caught when fishing with traditional fishing gear and if VHSV causes severe die-offs in herring fry, this would most likely not be known because the dead fish will quickly disappear in the aquatic system. It might therefore be that VHS plays a role in stock fluctuations in Atlantic herring populations without being recognized.

21.3.3 Transmission

Through studies of rainbow trout pathogenic virus isolates, transmission of VHSV has been shown to be horizontal through contact with other infected fish or contaminated water, etc. Virus is primarily shed from infected fish via the urine (Wolf, 1988). Recent studies using bioluminescence imaging of live trout infected with the closely related virus IHNV indicate that the skin plays an important role as virus uptake and multiplication on the base of

fish fins was very high (Harmache *et al.*, 2006). This remains to be examined for VHSV-infected fish. Recently, it was demonstrated that leeches may play an important role in disease transmission of VHSV type IVb, since the virus could be isolated from more than 50% of pools containing five leeches collected from fish in an endemically infected area (Faisal and Schulz, 2009). Piscivorous birds, such as herons, have been shown to contribute to the spread of virus between farms (Olesen and Vestergård Jørgensen, 1982; Peters and Neukirch, 1986).

Transmission readily occurs in the temperature range 1–15°C, but can occur up to 20°C. Incubation time is dependent on temperature and virus dose; it is 5–12 days at temperatures between 5 and 15°C – the lower the temperature the higher the incubation period. Virus excretion peaks a few days before the clinical signs and the first mortalities are observed at that point and go on for a few weeks hereafter. The excretion can last up to 3 months after infection at low temperatures below 3°C, while the fish will free themselves from the virus much faster at temperatures above 15°C (2–3 weeks) (Vestergård Jørgensen and Olesen, 1983).

True vertical transmission, i.e. intra ovum transmission, is unlikely to occur (Bovo *et al.*, 2005). Infected spawners can excrete large amounts of virus in ovarian fluid, but the eggs will tend to be free of virus after a few days in clean, flowing water (Jørgensen, 1970). Properly disinfected eggs should thus be considered a safe commodity.

21.3.4 Prevalence among wild fish

Since the discovery of VHSV strains in marine species, there have been a number of studies involving extensive sampling of a broad range of fish species from the coastal waters of continental Europe, the UK, North America and Asia (Skall *et al.*, 2005; Lee *et al.*, 2007). In these studies, samples were analysed for the presence of virus by inoculation onto fish cell lines or by PCR methods. In some studies, fish samples were pooled and therefore determination of precise prevalence was difficult. Nevertheless, based on virus isolation in cell culture, the prevalence of VHSV in the marine fish species sampled in these studies was in the range of 0–17% for Atlantic herring and sprat (*Sprattus sprattus*) in an area of the Baltic sea and 4–8% for sardine (*Sardinops sagax*), smelt (*Hypermesus pretiosus*) and mackerel (*Scomber japonicas*) off the coast of California and Oregon (Skall *et al.*, 2005). Marty *et al.* (2010) reported prevalence of VHSV at the rate of 0–27% in Pacific herring caught in Prince William Sound and Sitka Sound. From the Great Lakes region, Frattini *et al.* (2011) reported prevalence in VHSV-positive sites of 25–100% in emerald shiners (*Notropis artherinoides*) caught in Lake Erie and Niagara River and in bluntnose minnows (*Pimephales notatus*) from St Lawrence River. From the Black Sea, VHSV prevalence in whiting (*Merlangius merlangus*) from pooled samples was estimated to be 1.3–3.1%, whereas single samples showed prevalence up to 7%. In 1-year-old fish the prevalence was up to 25% (Altuntas and Ogut, 2010). Recently, Johansen *et al.* (2013) reported prevalence of 12–13% of Norwegian spring-spawning herring tested individually in the spawning season.

21.4 Diagnostics

The standard surveillance and diagnostic procedures for VHS have in the past five decades been based on isolation in fish cell cultures followed by immunochemical (IFAT, ELISA) or molecular (reverse transcription polymerase chain reaction (RT-PCR)) identification. As the direct identification of VHSV in fish tissue by immunochemical methods has a low sensitivity, these methods have not been applied for surveillance of the disease. Recently, however, two real-time RT-PCR assays have been developed and validated, proving similar sensitivity and specificity to the cell culture-based methods (Garver *et al.*, 2011; Jonstrup *et al.*, 2013) and these methods are expected to be implemented in future surveillance programmes.

21.4.1 Sampling

The sampling of fish for surveillance studies should be performed at an optimal time point when the water temperature is <14°C. All parts of the farm should be inspected for the presence of weak, abnormally behaving or dead fish. For both surveillance and diagnosis of VHS, the tissue material to be examined includes spleen, anterior kidney and either heart or encephalon. When sampling farms with broodstock, ovarian fluid may be examined as well. Organ pieces from a maximum of 10 fish may be pooled.

21.4.2 Cell cultivation

Virus can be isolated in various cell culture lines with kidney and spleen tissues yielding the highest viral titres in rainbow trout after an outbreak (Wolf, 1988). Detection of VHSV in clinically healthy carrier fish is more problematic (Skall *et al.*, 2005). In survivor rainbow trout, virus levels tend to be higher in the brain and the gill tissues than in the internal organs (Oidtmann *et al.*, 2011). In chronic-infected Pacific herring it was likewise found that titres in the brain were generally equal to or higher than titres in kidney and spleen tissues, and that the overall prevalence of infection was higher in the brain (Hershberger *et al.*, 2010).

Susceptibility of a cell line to infection will depend on a range of parameters, including cell-line lineage and viral strain differences; it thus appears that the bluegill fry (BF-2) cell line is the most sensitive to infection by genotype Ia strains, whereas the *epithelioma papulosum cyprini* (EPC) cell line may be more susceptible to VHSV genotype IV isolates than to type I, II or III isolates (Skall *et al.*, 2005). Among rainbow trout carriers of VHSV genotype Ia, using BF-2 cells detected twice as many positive fish as using EPC or Chinook salmon embryo (CHSE-214) cells (Olesen and Vestergård Jørgensen, 1992). Performing titrations, Lorenzen *et al.* (1999) reported that BF-2 and RTG-2 cell lines are equally sensitive and better than fathead minnow (FHM), CHSE-214 and EPC cells. Reports from the last five proficiency tests (PT) (2007–2011) issued by the European Union Reference Laboratory for Fish Diseases (http://www.eurl-fish.eu/Activities/proficiency-tests) showed, when comparing median titres of samples containing VHSV only, that in all the years the most susceptible cell line were BF-2 cells, followed by FHM and EPC cells, which in some years were as sensitive as BF-2 cells. RTG-2 cells were the least susceptible cell line in all the years except 2007.

Inoculated cells are incubated at 15°C and regularly examined for cytopathic effect (CPE). If no CPE is observed after 7–10 days, the samples are sub-cultivated onto new cells and examined for the next 7–10 days. If evidence of CPE has been observed in a cell culture, medium (supernatant) is collected and examined by one or more of the following techniques: neutralization, IFAT, ELISA, RT-PCR.

The exponential growth of virus in highly susceptible cell cultures makes this method very sensitive compared with methods not amplifying the virus. Another advantage of using cell cultures is that all known VHS viruses grow readily in cell cultures, making the diagnostic very robust, and when virus is first isolated it can easily be used for further characterization and studies. The major disadvantage is, however, the need for long incubation time (2 weeks) and for establishing highly specialized fish cell-culture facilities.

21.4.3 RT-PCR

Development and validation of RT-PCR assays with similar sensitivity and specificity towards all genotypes of VHSV was desired due to the fact that the cell culture-based methods have been a challenge. Therefore, recently two real-time RT-PCRs were developed demonstrating similar or higher sensitivity toward all known genotypes of VHSV (Garver *et al.*, 2011; Jonstrup *et al.*, 2013) and have been recognized as a substitute for cell

cultivation, giving the possibility of obtaining results of diagnosis and survey within a few hours.

21.4.4 Serology

Detection of VHSV-specific antibodies in farmed rainbow trout has been proposed as an alternative or supplement to the currently approved direct methods, because surveys based on serological tests have several advantages, especially in cases where water temperatures are too high for virus isolation and in endemically infected populations where there are no clinical signs of disease and the virus cannot be isolated in cell culture. A disadvantage of serological tests is that antibody cannot be detected earlier than 2 weeks to 3 months after infection, depending on the water temperature of the pond where the fish are cultured, with antibody developing faster at higher temperatures (Schyth *et al.*, 2012). Contrary to virus identification, a high antibody level following infection will last for at least 1 year, giving a much larger window for detecting exposure to VHS than virus isolation. The most widely used methods in VHS serology are seroneutralization, ELISA and IFAT. Seroneutralization is complement (C′) dependent, and therefore requires properly handled fish serum as a source of complement. Because C′ is quickly inactivated at temperatures above 20–30°C, the serum needs to be kept cold. In addition, the technique demands specialized cell culture facilities and long incubation periods of approximately 5 days. Seroneutralization is, however, very sensitive and specific and a robust method when first implemented (Olesen *et al.*, 1991; Fregeneda-Grandes and Olesen, 2007; Fregeneda-Grandes *et al.*, 2009). ELISA has also proven effective for antibody detection, but the sticky nature of the tetrameric fish immunoglobulin makes it prone to false positive reactions, therefore all fish sera examined should be used as its own negative control. ELISA has a sensitivity close to sero-neutralization, while its specificity is inferior. ELISA is the method of choice for mass screening in non-specialized laboratories. IFAT is efficient as well but more demanding for reading and interpretation and therefore less applied.

21.5 Vaccination

Fish surviving VHS infection develop a protective immune response and are much less susceptible to reinfection compared with naïve fish (Purcell *et al.*, 2012). Although research on vaccine development for VHS has been ongoing since the 1970s, a commercial vaccine is not yet available. Candidate vaccines have included killed vaccines, attenuated live vaccines, a recombinant vaccine in prokaryotic and eukaryotic expression systems, and DNA-based vaccines. The latter have proven to be very efficient and promising, inducing good protection against VHS, but have not been licensed. One of the problems is that only intramuscular delivery is possible, making it difficult to vaccinate fry before release into ponds (reviewed in Lorenzen and LaPatra, 2005).

21.6 Control Strategies and Zoosanitary Measures

VHS is listed as a reportable disease to the OIE and in the European Union it is listed as a 'non-exotic' disease and must be controlled accordingly. In order to prevent introduction and spreading of the disease into and between farms, a number of zoosanitary measures can be used. Netting over the ponds and fencing the sides of the farms is strongly advisable in order to prevent herons and other birds from gaining access. Spillage of water from fish transport lorries into the farm should be avoided. Fresh fish should not be used as feed for farmed fish. Transfer of live fish from sea water to fresh water is likewise not recommended.

Depending on local conditions, it is possible to eradicate the disease among farmed fish. In Denmark, the majority of fish farms (>400) were infected with the pathogen in the 1960s. Based on a programme agreed on between fish farmers and the authorities, the country succeeded in eradicating the disease, with the last outbreak taking place in January 2009. One of the key points in the programmes has been culling, followed by drainage and disinfection of the ponds and restocking after fallowing with certified virus-free fish (Fig. 21.3). Eradication procedures have been performed in the case of specific new outbreaks, but also in a more controlled way when all farms at the same system follow a predetermined plan (Olesen and Korsholm, 1997; Olesen, 1998).

21.7 Conclusions and Recommendations

From being known as a local disease affecting rainbow trout in European aquaculture, VHS has in recent years been recognized as a disease affecting a high number of both farmed and wild fish species all over the temperate northern hemisphere. Even though VHSV is one of the viral pathogens affecting fish that have been studied in most detail, precise knowledge of this virus and its effects is still lacking. Due to the distinct geographic distribution of VHSV, prevention of spreading isolates of different genotypes is an important issue and more research is needed in elucidating what makes an isolate pathogenic to one fish species but not to another. Finding these pathogenicity markers and being able to correlate them to the host will provide opportunities to optimize surveillance strategies and risk assessments regarding transfer of different types of VHSV.

VHS has been eradicated from several countries, both after single outbreaks and in endemically infected areas, but many countries are still struggling with VHS in fish farming, and are finding control measures and eradication difficult to apply. In these cases vaccination would be a usable tool in combating the disease. Furthermore, because it will never be possible to eradicate this disease among the wildlife, there will always be a risk for farmed fish. In areas where this risk is enhanced, it may be practical to vaccinate the farmed fish. Despite many years of research within this field, a VHS vaccine is still not available on the market.

Fig. 21.3. Ponds approximately 1 week after drainage and disinfection with quicklime. The ponds should be left empty for at least 6 weeks during the summer period before restocking.

References

Altuntas, C. and Ogut, H. (2010) Monthly occurrence and prevalence of viral haemorrhagic septicaemia virus (VHSV) in whiting *Merlangius merlangus*. *Diseases of Aquatic Organisms* 88, 107–113.

Ariel, E. and Olesen, N.J. (2002) Finfish in aquaculture and their diseases – a retrospective view on the European Community. *Bulletin of the European Association of Fish Pathologists* 22, 72–85.

Bearzotti, M., Delmas, B., Lamoureux, A., Loustau, A.M., Chilmonczyk, S. and Bremont, M. (1999) Fish rhabdovirus cell entry is mediated by fibronectin. *Journal of Virology* 73, 7703–7709.

Bovo, G., Håstein, T., Hill, B., LaPatra, S., Michel, C., Olesen, N.J., Shchelkunov, I., Storset, A., Wollfrom, T. and Midtlying, P.J. (2005) QLK2-CT-2002-01546: Fish Egg Trade Work package 1 report: hazard identification for vertical transfer of fish disease agents. Pp. 1–35.

Brudeseth, B.E., Castric, J. and Evensen, Ø. (2002) Studies on pathogenesis following single and double infection with viral hemorrhagic septicemia virus and infectious hematopoietic necrosis virus in rainbow trout (*Oncorhynchus mykiss*). *Veterinary Pathology* 39, 180–189.

Brunson, R., True, K. and Yancey, J. (1989) VHS virus isolated at Makah National Fish Hatchery. *American Fisheries Society Fish Health Section Newsletter* 17, 3–4.

Dale, O.B., Ørpetveit, I., Lyngstad, T.M., Kahns, S., Skall, H.F., Olesen, N.J. and Dannevig, B.H. (2009) Outbreak of viral haemorrhagic septicaemia (VHS) in seawater-farmed rainbow trout in Norway caused by VHS virus Genotype III. *Diseases of Aquatic Organisms* 85, 93–103.

de Kinkelin, P. and Le Berre, M. (1977) Isolement d'un Rhabdovirus pathogéne de la Truite Fario (*Salmo trutta* L., 1766). *Comptes Rendus de l'Académie des Science Paris* 284, 101–104.

Dietzgen, R.G., Calisher, C.H., Kurath, G., Kuzmin, I.V., Rodriguez, L.L., Stone, D.M., Tesh, R.B., Tordo, N., Walker, P.J., Wetzel, T. and Whitfield, A.E. (2012) Family Rhabdoviridae. In: King, A.M.Q., Adams, M.J., Carstens, E.B. and Lefkowitz, E.J. (eds) *Virus Taxonomy*. Elsevier Academic Press, Amsterdam, pp. 686–713.

Einer-Jensen, K., Ahrens, P., Forsberg, R. and Lorenzen, N. (2004) Evolution of the fish rhabdovirus viral haemorrhagic septicaemia virus. *Journal of General Virology* 85, 1167–1179.

Einer-Jensen, K., Ahrens, P. and Lorenzen, N. (2005a) Parallel phylogenetic analyses using the N, G or Nv gene from a fixed group of VHSV isolates reveal the same overall genetic typing. *Diseases of Aquatic Organisms* 67, 39–45.

Einer-Jensen, K., Winton, J. and Lorenzen, N. (2005b) Genotyping of the fish rhabdovirus, viral haemorrhagic septicaemia virus, by restriction fragment length polymorphisms. *Veterinary Microbiology* 106, 167–178.

Elsayed, E., Faisal, M., Thomas, M., Whelan, G., Batts, W. and Winton, J. (2006) Isolation of viral haemorrhagic septicaemia virus from muskellunge, *Esox masquinongy* (Mitchill), in Lake St Clair, Michigan, USA reveals a new sublineage of the North American genotype. *Journal of Fish Diseases* 29, 611–619.

Enzmann, P.J. and Konrad, M. (1985) Inapparent infections of brown trout with VHS-virus. *Bulletin of the European Association of Fish Pathologists* 5, 81–83.

Faisal, M. and Schulz, C.A. (2009) Detection of Viral Hemorrhagic Septicemia virus (VHSV) from the leech *Myzobdella lugubris* Leidy, 1851. *Parasites & Vectors* 2, 45.

Faisal, M., Shavalier, M., Kim, R.K., Millard, E.V., Gunn, M.R., Winters, A.D., Schulz, C.A., Eissa, A., Thomas, M.V., Wolgamood, M., Whelan, G.E. and Winton, J. (2012) Spread of the emerging viral hemorrhagic septicemia virus strain, genotype IVb, in Michigan, USA. *Viruses* 4, 734–760.

Frattini, S.A., Groocock, G.H., Getchell, R.G., Wooster, G.A., Casey, R.N., Casey, J.W. and Bowser, P.R. (2011) A 2006 survey of viral hemorrhagic septicemia (VHSV) virus type IVb in New York State waters. *Journal of Great Lakes Research* 37, 194–198.

Fregeneda-Grandes, J.M. and Olesen, N.J. (2007) Detection of rainbow trout antibodies against viral haemorrhagic septicaemia virus (VHSV) by neutralisation test is highly dependent on the virus isolate used. *Diseases of Aquatic Organisms* 74, 151–158.

Fregeneda-Grandes, J.M., Skall, H.F. and Olesen, N.J. (2009) Antibody response of rainbow trout with single or double infections involving viral haemorrhagic septicaemia virus and infectious haematopoietic necrosis virus. *Diseases of Aquatic Organisms* 83, 23–29.

Gadd, T., Jakava-Viljanen, M., Einer-Jensen, K., Ariel, E., Koski, P. and Sihvonen, L. (2010) Viral haemorrhagic septicaemia virus (VHSV) genotype II isolated from European river lamprey *Lampetra fluviatilis* in Finland during surveillance from 1999 to 2008. *Diseases of Aquatic Organisms* 88, 189–198.

Gadd, T., Jakava-Viljanen, M., Tapiovaara, H., Koski, P. and Sihvonen, L. (2011) Epidemiological aspects of viral haemorrhagic septicaemia virus genotype II isolated from Baltic herring, *Clupea harengus membras* L. *Journal of Fish Diseases* 34, 517–529.

Gagné, N., MacKinnon, A.-M., Boston, L., Souter, B., Cook-Versloot, M., Griffiths, S. and Olivier, G. (2007) Isolation of viral haemorrhagic septicaemia virus from mummichog, stickleback, striped bass and brown trout in eastern Canada. *Journal of Fish Diseases* 30, 213–223.

Garver, K.A., Hawley, L.M., McClure, C.A., Schroeder, T., Aldous, S., Doig, F., Snow, M., Edes, S., Baynes, C. and Richard, J. (2011) Development and validation of a reverse transcription quantitative PCR for universal detection of viral hemorrhagic septicemia virus. *Diseases of Aquatic Organisms* 95, 97–112.

Harmache, A., LeBerre, M., Droineau, S., Giovannini, M. and Bremont, M. (2006) Bioluminescence imaging of live infected salmonids reveals that the fin bases are the major portal of entry for Novirhabdovirus. *Journal of Virology* 80, 3655–3659.

Hedrick, R.P., Batts, W.N., Yun, S., Traxler, G.S., Kaufman, J. and Winton, J.R. (2003) Host and geographic range extensions of the North American strain of viral hemorrhagic septicemia virus. *Diseases of Aquatic Organisms* 55, 211–220.

Hershberger, P.K., Gregg, J.L., Grady, C.A., Taylor, L. and Winton, J.R. (2010) Chronic and persistent viral hemorrhagic septicemia virus infections in Pacific herring. Diseases of Aquatic Organisms 93, 43–49.

Hopper, K. (1989) The isolation of VHSV from chinook salmon at Glenwood Springs, Orcas Island, Washington. *American Fisheries Society Fish Health Section Newsletter* 17, 1.

Ito, T., Olesen, N.J., Skall, H.F., Sano, M., Kurita, J., Nakajima, K. and Iida, T. (2010) Development of a monoclonal antibody against viral haemorrhagic septicaemia virus (VHSV) genotype IVa. *Diseases of Aquatic Organisms* 89, 17–27.

Ito, T., Kurita, J., Skall, H.F., Lorenzen, N. and Olesen, N.J. (2011) P-162: Viral haemorrhagic septicaemia virus (VHSV) in rainbow trout: virulence variability within genotype Ib isolates. *Abstract Book – 15th EAFP International Conference on Diseases of Fish and Shellfish*, 337.

Ito, T., Kurita, J., Sano, M., Skall, H.F., Lorenzen, N., Einer-jensen, K. and Olesen, N.J. (2012) Typing of viral hemorrhagic septicemia virus by monoclonal antibodies. *Journal of General Virology* 93, 2546–2557.

Jensen, M.H. (1963) Preparation of fish tissue cultures for virus research. *Bulletin de l'Office des International Epizooties* 59, 131–134.

Jensen, M.H. (1965) Research on the virus of Egtved disease. *Annals of the New York Academy of Sciences* 126, 422–426.

Jensen, N.J., Bloch, B. and Larsen, J.L. (1979) The ulcus-syndrome in cod (*Gadus morhua*) III. A preliminary virological report. *Nordisk Veterinærmedicin* 31, 436–442.

Johansen, R., Bergh, O., Modahl,I., Dahle, G., Gjerset, B., Holst, J.C. and Sandlund, N. (2013) High prevalence of viral haemorragic septicaemia virus (VHSV) in Norwegian spring-spawning herring. Marine Ecology Progress Series U78, 223–230.

Jonstrup, S.P., Gray, T., Kahns, S., Skall, H.F., Snow, M. and Olesen, N.J. (2009) FishPathogens.eu/vhsv: a user-friendly viral haemorrhagic septicaemia virus isolate and sequence database. *Journal of Fish Diseases* 32, 925–929.

Jonstrup, S.P., Kahns, S., Skall, H.F., Boutrup, T.S. and Olesen, N.J. (2013) Development and validation of a novel Taqman-based real-time RT-PCR assay suitable for demonstrating freedom from viral haemorrhagic septicaemia virus. *Journal of Fish Diseases*, 36, 9–23.

Jørgensen, P.E.V. (1970) The survival of viral haemorrhagic septicaemia (VHS) virus associated with trout eggs. *Rivista Italiana di Piscicoltura e Ittiopatologia* 1, 13–15.

Jørgensen, P.E.V. (1982) Egtved virus: occurrence of inapparent infections with virulent virus in free-living rainbow trout, *Salmo gairdneri* Richardson, at low temperature. *Journal of Fish Diseases* 5, 251–255.

Kahns, S., Skall, H.F., Kaas, R.S., Korsholm, H., Bang Jensen, B., Jonstrup, S.P., Dodge, M.J., Einer-Jensen, K., Stone, D. and Olesen, N.J. (2012) European freshwater VHSV genotype Ia isolates divide into two distinct subpopulations. *Diseases of Aquatic Organisms* 99, 23–35.

Kalayci, G., Incoglu, S. and Özkan, B. (2006) First isolation of viral haemorrhagic speticaemia (VHS) virus from turbot (*Scopthalmus maximus*) cultures in the Trabzon coastal area of the Black Sea in Turkey. *Bulletin of the European Association of Fish Pathologists* 26, 157–162.

Kim, S.-M., Lee, J.-I., Hong, M.-J., Park, H.-S. and Park, S.-I. (2003) Genetic relationship of the VHSV (viral hemorrhagic septicemia virus) isolated from cultured olive flounder, *Paralichthys olivaceus* in Korea. *Journal of Fish Pathology* 16, 1–12.

Kocan, R., Bradley, M., Elder, N., Meyers, T., Batts, W. and Winton, J. (1997) North American strain of viral hemorrhagic septicemia virus is highly pathogenic for laboratory-reared Pacific herring. *Journal of Aquatic Animal Health* 9, 279–290.

Lee, W.-L., Yun, H.-M., Kim, S.-R., Jung, S.-J. and Oh, M.-J. (2007) Detection of viral hemorrhagic septicemia virus (VHSV) from marine fish in the south western coastal area and East China Sea. *Journal of Fish Pathology* 20, 201–209.

López-Vázquez, C., Raynard, R.S., Bain, N., Snow, M., Bandín, I. and Dopazo, C.P. (2006) Genotyping of marine viral haemorrhagic septicaemia virus isolated from the Flemish Cap by nucleotide sequence analysis and restriction fragment length polymorphism patterns. *Diseases of Aquatic Organisms* 73, 23–31.

Lorenzen, E., Carstensen, B. and Olesen, N.J. (1999) Inter-laboratory comparison of cell lines for susceptibility to three viruses: VHSV, IHNV and IPNV. *Diseases of Aquatic Organisms* 37, 81–88.

Lorenzen, N. and LaPatra, S.E. (2005) DNA vaccines for aquacultured fish. *Scientific and Technical Review* 24, 201–213.

Lorenzen, N., Olesen, N.J. and Jorgensen, P.E.V. (1988) Production and characterization of monoclonal antibodies to four Egtved virus structural proteins. *Diseases of Aquatic Organisms* 4, 35–42.

Marty, G.D., Hulson, P.-J.F., Miller, S.E., Quinn II, T.J., Moffit, S.D. and Merizon, R.A. (2010) Failure of population recovery in relation to disease in Pacific herring. *Diseases of Aquatic Organisms* 90, 1–14.

Meyers, T.R. and Winton, J.R. (1995) Viral hemorrhagic septicemia virus in North America. *Annual Review of Fish Diseases* 5, 3–24.

Nishizawa, T., Iida, H., Takano, R., Isshiki, T., Nakajima, K. and Muroga, K. (2002) Genetic relatedness among Japanese, American and European isolates of viral hemorrhagic septicemia virus (VHSV) based on partial G and P genes. *Diseases of Aquatic Organisms* 48, 143–148.

Nishizawa, T., Savas, H., Isidan, H., Üstündag, C., Iwamoto, H. and Yoshimizu, M. (2006) Genotyping and pathogenicity of viral hemorrhagic septicemia virus from free-living turbot (*Psetta maxima*) in a Turkish coastal area of the Black Sea. *Applied and Environmental Microbiology* 72, 2373–2378.

Nordblom, B. (1998) *Report on an Outbreak of Viral Haemorrhagic Septicaemia in Sweden*. Swedish Board of Agriculture, Jönköping, Sweden.

Nordblom, B. and Norell, A.W. (2000) *Report on an Outbreak of VHS (Viral Hemorrhagic Septicaemia) in Farmed Fish in Sweden*. Report for the Standing Veterinary Committee. Swedish Board of Agriculture, Jönköping, Sweden.

Oidtmann, B., Joiner, C., Stone, D., Dodge, M., Reese, R.A. and Dixon, P. (2011) Viral load of various tissues of rainbow trout challenged with viral haemorrhagic septicaemia virus at various stages of disease. *Diseases of Aquatic Organisms* 93, 93–104.

OIE (2009) Viral haemorrhagic septicaemia. In: *Manual of Diagnostic Tests for Aquatic Animals*, pp. 279–298 (http://www.oie.int/fileadmin/Home/eng/Health_standards/aahm/2010/2.3.09.VHS.pdf).

Olesen, N.J. (1998) Sanitation of viral haemorrhagic septicaemia (VHS). *Journal of Applied Ichthyology – Zeitschrift für Angewandte Ichthyologie* 14, 173–177.

Olesen, N.J. and Korsholm, H. (1997) Control measures for viral diseases in aquaculture: eradication of VHS and IHN. *Bulletin of the European Association of Fish Pathologists* 17, 229–233.

Olesen, N.J. and Vestergård Jørgensen, P.E. (1982) Can and do herons serve as vectors for Egtved virus? *Bulletin of the European Association of Fish Pathologists* 2, 48.

Olesen, N.J. and Vestergård Jørgensen, P.E. (1992) Comparative susceptibility of three fish cell lines to Egtved virus, the virus of viral haemorrhagic septicaemia (VHS). *Diseases of Aquatic Organisms* 12, 235–237.

Olesen, N.J., Lorenzen, N. and Jørgensen, P.E.V. (1991) Detection of rainbow trout antibody to Egtved virus by enzyme-linked immunosorbent assay (ELISA), immunofluorescence (IF), and plaque neutralization tests (50%PNT). *Diseases of Aquatic Organisms* 10, 31–38.

Olesen, N.J., Lorenzen, N. and Jørgensen, P.E.V. (1993) Serological differences among isolates of viral haemorrhagic septicaemia virus detected by neutralizing monoclonal and polyclonal antibodies. *Diseases of Aquatic Organisms* 16, 163–170.

Peters, F. and Neukirch, M. (1986) Transmission of some fish pathogenic viruses by the heron, *Ardea cinerea*. *Journal of Fish Diseases* 9, 539–544.

Pierce, L.R. and Stepien, C.A. (2012a) Evolution and biogeography of an emerging quasispecies: diversity patterns of the fish Viral Hemorrhagic Septicemia virus (VHSv). *Molecular Phylogenetics and Evolution* 63, 327–341.

Pierce, L.R. and Stepien, C.A. (2012b) Corrigendum to: 'Evolution and biogeography of an emerging quasispecies: Diversity patterns of the fish Viral Hemorrhagic Septicemia virus (VHSv)' [*Mol. Phylogenet. Evol*. 63 (2012) 327–347]. *Molecular Phylogenetics and Evolution* 65, 349.

Purcell, M.K., Laing, K.J. and Winton, J.R. (2012) Immunity to fish rhabdoviruses. *Viruses* 4, 140–166.

Raja-Halli, M., Vehmas, T.K., Rimaila-Parnanen, E., Sainmaa, S., Skall, H.F., Olesen, N.J. and Tapiovaara, H. (2006) Viral haemorrhagic septicaemia (VHS) outbreaks in Finnish rainbow trout farms. *Diseases of Aquatic Organisms* 72, 201–211.

Schäperclaus, W. (1938) Die schädigungen der deutschen fischerei durch fischparasiten und fishchkrankheiten. *Fischerei-Zeitung* 22, 1–20.

Schlotfeldt, H.J., Ahne, W., Vestergård-Jørgensen, P.E. and Glende, W. (1991) Occurrence of viral haemorrhagic septicaemia in turbot (*Scophthalmus maximus*) – a natural outbreak. *Bulletin of the European Association of Fish Pathologists* 11, 105–107.

Schütze, H., Mundt, E. and Mettenleiter, T.C. (1999) Complete genomic sequence of viral hemorrhagic septicemia virus, a fish rhabdovirus. *Virus Genes* 19, 59–65.

Schyth, B.D., Ariel, E., Korsholm, H. and Olesen, N.J. (2012) Diagnostic capacity for viral haemorrhagic septicaemia virus (VHSV) infection in rainbow trout (*Oncorhynchus mykiss*) is greatly increased by combining viral isolation with specific antibody detection. *Fish and Shellfish Immunology* 32, 593–597.

Skall, H.F., Slierendrecht, W.J., King, J.A. and Olesen, N.J. (2004) Experimental infection of rainbow trout *Oncorhynchus mykiss* with viral haemorrhagic septicaemia virus isolates from European marine and farmed fishes. *Diseases of Aquatic Organisms* 58, 99–110.

Skall, H.F., Olesen, N.J. and Mellergaard, S. (2005) Viral haemorrhagic septicaemia virus in marine fish and its implications for fish farming – a review. *Journal of Fish Diseases* 28, 509–529.

Smail, D.A. (1995) *Isolation and Identification of Viral Haemorrhagic Septicaemia (VHS) Virus from North Sea Cod* (Gadus morhua L.). *CM 1995/F:5*. International Council for Exploration of the Seas, Copenhagen.

Smail, D.A. (2000) Isolation and identification of viral haemorrhagic septicaemia (VHS) viruses from cod *Gadus morhua* with the ulcus syndrome and from haddock *Melanogrammus aeglefinus* having skin haemorrhages in the North Sea. *Diseases of Aquatic Organisms* 41, 231–235.

Snow, M., Cunningham, C.O., Melvin, W.T. and Kurath, G. (1999) Analysis of the nucleoprotein gene identifies distinct lineages of viral haemorrhagic septicaemia virus within the European marine environment. *Virus Research* 63, 35–44.

Snow, M., Bain, N., Black, J., Taupin, V., Cunningham, C.O., King, J.A., Skall, H.F. and Raynard, R.S. (2004) Genetic population structure of marine viral haemorrhagic septicaemia virus (VHSV). *Diseases of Aquatic Organisms* 61, 11–21.

Stone, D.M., Kerr, R.C. and Darby, A.C. (2011) P-193: The complete genome sequence of four putative vesiculoviruses, tench rhabdovirus (TENRV), grass carp rhabdovirus (GRCRV), perch rhabdovirus (PRV) and eel rhabdovirus (EVEX). *Abstract Book – 15th EAFP International Conference on Diseases of Fish and Shellfish*, p. 368.

Thompson, T.M., Batts, W.N., Faisal, M., Bowser, P., Casey, J.W., Phillips, K., Garver, K.A., Winton, J. and Kurath, G. (2011) Emergence of viral hemorrhagic septicemia virus in the North American Great Lakes region is associated with low viral genetic diversity. *Diseases of Aquatic Organisms* 96, 29–43.

Toplak, I., Hostnik, P., Rihtarič, D., Olesen, N.J., Skall, H.F. and Jenčič, V. (2010) First isolation and genotyping of viruses from recent outbreaks of viral haemorrhagic septicaemia (VHS) in Slovenia. *Diseases of Aquatic Organisms* 92, 21–29.

USDA-APHIS-VS (2008) Species regulated by title 9 CFR Parts 83.1 through 83.7, 93.900 and 93.910 through 93.916 (the Viral Hemorrhagic Septicemia (VHS) Interim Rule). (http://www.aphis.usda.gov/animal_health/animal_dis_spec/aquaculture/downloads/vhs_regulated_spp.pdf).

Vestergård Jørgensen, P.E. and Olesen, N.J. (1983) In: *Bekæmpelse af Egtvedsyge (VHS) hos regnbueørreder*. En artikelserie. Fællesudvalget for det Danske Ørrederhverv, Herning, pp. 1–56.

Vestergård Jørgensen, P.E. and Olesen, N.J. (1987) Cod ulcus syndrome rhabdovirus is indistinguishable from the Egtved (VHS) virus. *Bulletin of the European Association of Fish Pathologists* 7, 73–74.

Winton, J., Kurath, G. and Batts, W. (2008) Molecular epidemiology of viral hemorrhagic septicemia virus in the Great Lakes region. U.S. Department of the Interior, U.S. Geological Survey *USGS FS* 2008–2003.

Wolf, K. (1988) Viral hemorrhagic septicaemia. In: *Fish Viruses and Fish Viral Diseases*. Cornell University Press, Ithaca, New York, pp. 217–249.

22 Snakehead Rhabdovirus

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22.1 Introduction and Overview

The focus of this chapter is snakehead rhabdovirus (SHRV), a Novirhabdovirus, in the family *Rhabdoviridae*. We will present a history of this virus in the natural host and provide an overview of the current state of knowledge about this virus family, with particular focus on SHRV. Rhabdoviruses have a wide host range and can infect plants, insects and vertebrates, including humans and other mammals. A number of rhabdoviruses are pathogens that are responsible for causing devastating economic losses to the finfish industry. Several rhabdovirus species infect fish (see Table 22.1). In our laboratory, we are using the SHRV infection of zebrafish (*Danio rerio*) to model fish rhabdoviral infections and the subsequent host innate immune response. With the molecular reagents, transgenic animals and genomic data available for the zebrafish system, this model species can provide critical insight into the mechanisms of viral pathogenesis.

SHRV was isolated from striped snakehead fish during an outbreak of epizootic ulcerative syndrome (EUS) (Ahne *et al*., 1988; Wattanavijarn *et al*., 1986). EUS is a seasonal, freshwater and estuarine disease of ambiguous aetiology that has been identified in numerous fish species (>100) from Asia, Australia, North America and Africa. Outbreaks of EUS have had dramatic impacts on regional fisheries, threatening local economies and food supplies (Oidtmann, 2012). EUS typically manifests itself in affected fish through skin lesions that lead to subsequent infection of underlying musculature and viscera. While the exact mode(s) of infection remain controversial, it is generally accepted that fish afflicted with EUS retain a common pathological feature of ulcerative lesions containing fungal hyphae (usually the ubiquitous *Aphanomyces invadans*, also known as *A. piscida*) that elicit granulomatous responses (Baldock *et al*., 2005). The most widely held belief is that *A. invadans* is the primary causative agent of EUS outbreaks and that these infections are thought to be facilitated by environmental factors, such as infection by viruses like SHRV or cutaneous damage. Some investigators hold that EUS represents a constellation of polymicrobial infections involving viruses (like SHRV), bacteria and fungi, including, but not always, *A. invadans*, that are triggered by environmental insults (e.g. pH, temperature, salinity, mechanical, infection) (Baldock *et al*., 2005; Oidtmann, 2012). It is critical to emphasize that while viruses like SHRV are

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Table 22.1. Fish rhabdoviruses. Selected references (1986–2010) on rhabdoviruses that afflict fish species. Members of the *Vesiculovirus* genus and the *Novirhabdovirus* genus are shown.

Genus	Virus	Known susceptible hosts	References
Novirhabdovirus	Snakehead rhabdovirus (SHRV)	Snakehead	Altmann *et al*. (2003, 2004), Alonso *et al*. (2004), Hermann *et al*. (2004), Phelan *et al*. (2005a, 2005b), Nayak *et al*. (2007)
	Infectious haematopoietic necrosis virus (IHNV)	Salmon, trout	Chiou *et al*. (2000), Romero *et al*. (2005)
	Hirame rhabdovirus (HIRRV)	Japanese flounder, ayu, mebaru, black seabream	Nishizawa *et al*. (1991a, 1991b)
	Viral haemorrhagic septicaemia virus (VHSV)	Salmonid fishes, rainbow trout	Novoa *et al*. (2006), Encinas *et al*. (2010)
	Pike fry rhabdovirus (PFRV)	Pike, grass carp	Lilley and Frerichs (1994), Hoffman *et al*. (2005)
Vesiculovirus	Starry flounder virus (SFRV)	Flounder	Mork *et al*. (2004)
	Ulcerative disease rhabdovirus (UDRV)	Snakehead, climbing perch, Nile tilapia, grass carp	Frerichs *et al*. (1986), Kasornchandra *et al*. (1992a)
	Swedish sea trout rhabdovirus (SSTV)	Sea trout	Johansson *et al*. (2002)
	Spring viremia of carp virus (SVCV)	Cyprinid fishes	Sanders *et al*. (2003), Wang *et al*. (2006), Lopez-Munoz *et al*. (2010)
	Eel virus Europe X (EVEX)	Eel	Hoffman *et al*. (2005)
	Eel virus American (EVA)	Eel	Hoffman *et al*. (2005)

thought to participate to some degree in EUS, they are not the principal causative agent.

Despite this, many previously undescribed viruses (Frerichs *et al*., 1986; Hedrick *et al*., 1986; Ahne *et al*., 1988; Wattanavijarn *et al*., 1988; Subramaniam *et al*., 1993; Roberts *et al*., 1994; Frerichs, 1995; John *et al*., 2001) have been isolated from fish during EUS outbreaks, and these have provided critical insights into fish immunology.

22.1.1 History: isolation of rhabdovirus, including SHRV

Numerous rhabdoviruses have been isolated from EUS-affected fish. Frerichs *et al*. (1986) was the first to report a single virus type that had been isolated from multiple species of fish across a broad geographic area (Thailand and Burma). This virus, derived from wild and pond-culture striped snakehead fish, as well as freshwater eels, formed a bullet-shaped particle with a typical rhabdoviral structure (120 ± 10 nm × 80 ± 5 nm). The virus was subsequently identified as ulcerative disease rhabdovirus (UDRV). Ahne *et al*. (1988) later characterized a similar rhabdovirus that had been isolated from striped snakehead fish by Wattanavijarn and Wattanodorn (1986) following an EUS outbreak in Thailand. This virus came to be known as SHRV, and serological analysis revealed no relationship to five other reference fish rhabdoviruses. Kasornchandra *et al*. (1992a) undertook a more intensive comparative study of the rhabdoviruses isolated by Wattanavijarn *et al*. (1986) and Frerichs *et al*. (1986, 1989). SHRV was found to have a bacilliform morphology and ranged in size from 180 to 200 nm long and 60 to 70 nm wide (Kasornchandra *et al*., 1992a). An analysis of the structural proteins by SDS-PAGE revealed a pattern similar to infectious haematopoietic necrosis virus (IHNV), viral haemorrhagic

septicaemia virus (VHSV) and hirame rhabdovirus (HIRRV) (Kasornchandra *et al.*, 1992a). The two UDRV particles that were characterized (UDRV-BP and UDRV-19) were found to have bullet-shape morphology and ranged in size from 110 to 130 nm long and 50 to 65 nm wide. SDS-PAGE revealed that the structural proteins possessed by UDRV-BP and UDRV-19 isolates were most similar to spring viremia of carp virus (SVCV) and pike fry rhabdovirus (PFRV) (Kasornchandra *et al.*, 1992a). Serological comparisons demonstrated that while they were both isolated from snakehead fish, SHRV and the UDRV viruses were distinct rhabdoviruses, although UDRV-19 and UDRV-BP appeared to be identical.

Subsequently, Lilley and Frerichs (1994) characterized a novel rhabdovirus, known as T9204, which had been isolated from striped snakehead. While bearing close similarity to SHRV, it differed in several ways and thus was determined to be another novel rhabdovirus strain associated with EUS. Lio-Po *et al.* (2000) identified 91-97 as yet another rhabdoviral isolate from samples collected from snakehead during an EUS outbreak in the Philippines in 1991. The 91-97 virus exhibited different serological properties, which indicated that it was antigenically distinct from SHRV and more closely related to UDRV.

22.2 Snakehead Rhabdovirus Genome Structure and Life Cycle

SHRV replicates at an optimum temperature range of 24–30°C. SHRV, like other rhabdoviruses, is an enveloped, nonsegmented, negative-sense, single-stranded RNA virus. The virus is built around the negative-sense RNA genome, which is approximately 11,000 nucleotides (11 kb) long. Rhabdoviruses in general have five genes that are organized in a linear arrangement. Novirhabdoviruses are unique in that they have six genes, with an additional nonvirion (NV) gene between the glycoprotein (G) and polymerase (L). The small genome has an unmodified 5′-triphosphate (5′-ppp) group and 3′ hydroxyl ends and is comprised of genes with the gene order (3′-N-P-M-G-NV-L-5′). The first four nucleotides at the 3′-ends of SHRV, IHNV, HIRRV and VHSV are identical (Kim *et al.*, 2005). The gene order is conserved among Rhabdoviruses and between the genes there are short intergenic regions (Purcell *et al.*, 2012). Briefly, the nucleoprotein (N) encloses the RNA; the phosphoprotein (P) is a co-factor for the RNA polymerase and a chaperone for N; the matrix (M) protein is critical for virus assembly and budding; the G is a transmembrane protein responsible for attachment to target cells and facilitates membrane fusion; and the L is the viral RNA polymerase (Rieder and Conzelmann, 2011). Among fish rhabdoviruses, the L protein of SHRV shows the highest sequence homology to HIRRV, IHNV and VHSV (Kim *et al.*, 2005). The ratio of gene expression is regulated during transcription. For instance, there is a polarity of transcription that starts at the 3′ end of the virus genome to the 5′ end, and results in more mRNA for proteins encoded in the 3′ end of the genome than for proteins located at the 5′ end (Cann, 1993; Rieder and Conzelmann, 2011).

The entire lifecycle of SHRV occurs in the cytoplasm of the infected cell and replication is carried out by the viral RNA polymerase. Packaging of this enzyme within the virus nucleocapsid is essential because no known hosts contain enzymes capable of decoding and copying the RNA genome (Cann, 1993). As a negative-sense RNA virus, it contains virion RNA that is complementary to mRNA, which must be copied into the complementary positive sense mRNA before viral proteins can be made. The newly transcribed negative strands may be used as templates for a full-length negative-sense genome.

SHRV enters its target cell via receptor-mediated endocytosis. This process is mediated by interaction of the viral G protein with a cell surface receptor. The G protein is synthesized by ribosomes bound to the endoplasmic reticulum and is transported and inserted into the plasma membrane. These trimeric proteins are critical for assembly into virions, and virus entry, because G proteins promote fusion of the viral and host endosomal membranes, after which the viral

nucleocapsid is released into the cytoplasm of the host cell.

22.3 Phylogeny of Rhabdoviruses

Fish rhabdoviruses were first assigned to either the genus *Vesiculovirus* or the genus *Lyssavirus* based on their protein profiles following gel electrophoresis. Mammalian viruses such as rabies virus and vesicular stomatitis virus also fall into these genera. Additional accepted genera include: *Ephemerovirus*, *Cytorhabdovirus*, *Nucleorhabdovirus* and *Novirhabdovirus* (Hoffman *et al.*, 2005). Molecular phylogenies have been determined for the *Rhabdoviridae* family using N, G or L gene sequences. It should be noted that a general phylogenic tree cannot be constructed based on the G gene due to low sequence identities, and sequences for the L gene are not available for all of the species of each genus.

A phylogenetic tree of the Rhabdoviridae family has been constructed using sequences of the N gene, which is reasonably conserved (Hoffman *et al.*, 2005). The phylogenetic analysis confirmed the established classification of rhabdoviruses into six genera. Based on complete N gene sequences, fish viruses fall within the *Vesiculovirus* and *Novirhabdovirus* genera (Hoffman *et al.*, 2005) (see also Table 22.1). Species within the *Novirhabdovirus* genus contain nucleotide sequences that are similar among all identified members of the genus (e.g. phylogenetic clustering), but are distinct from rhabdoviruses of the other genera (Hoffman *et al.*, 2005). Of interest, all members of this genus are fish pathogens. The gene is conserved among all of the virus species within this genus, implying that NV may play a significant role in viral pathogenesis (also see section 22.5).

22.4 Pathogenesis: Clinical Symptoms

Fish rhabdoviruses, in general, cause disease that is commonly characterized by acute haemorrhagic septicaemia (Purcell *et al.*, 2012). The disease manifestation impacts multiple organs and clinical symptoms such as skin darkening, ascites and exopthalmia can be observed, although it should be noted that varied forms of disease have been described for IHNV and VHSV (Purcell *et al.*, 2012).

22.4.1 Clinical symptoms of EUS

As mentioned above, SHRV has been associated with EUS (Wattanavijarn *et al.*, 1986; Ahne *et al.*, 1988). Upon further characterization, researchers have found that EUS is a complex disease process that seems to involve mechanical, environmental and pathological insults. Oidtmann (2012) provides a thorough review of the clinical and diagnostic features of EUS. Briefly, fish affected by EUS develop clinical signs that are often indistinguishable from other conditions. Typical EUS symptoms are non-specific in nature and range from mild reddening of the skin to severe ulcerations. Histopathologically, fish exhibiting signs of EUS often, but not always, form mycotic epithelial granulomas as a result of an infection of the muscle tissue. In some circumstances, the presence of multinucleated giant cells, in the context of other factors, are indicative of EUS. In other instances, EUS fish succumb to infections prior to the display of these signs of a robust inflammatory response. These EUS clinical symptoms differ from those observed in experimental SHRV infections in zebrafish, in which discrete pathological features were observed, and in snakehead fish, where no obvious EUS-like lesions were noted following challenge by immersion or intraperitoneal injection. SHRV may contribute to EUS but does not itself cause EUS. The clinical symptoms observed are triggered by infection with *A. invadans*.

22.5 Pathogenesis and Clinical Symptoms: SHRV

Our laboratory has previously reported the successful infection of zebrafish embryos and adults with SHRV (Phelan *et al.*, 2005b).

Pathological effects were observed in zebrafish infected with SHRV, and the infection elicited an antiviral innate immune response (discussed in detail in section 22.6). Experimental infection of both embryonic and adult zebrafish led to infection kinetics and histopathology indicative of acute infection.

Gross pathology was observed in adult zebrafish injected intraperitoneally with SHRV. Fish were examined 2 days post infection with SHRV for signs of viral infection and clinical disease. Moribund fish showed erratic swimming patterns and lingered near the surface of the water. Adults also exhibited severe petechial haemorrhages on the abdomen compared with control fish, which exhibited no erratic behaviour or lesions (Phelan *et al.*, 2005b).

Histological examination was conducted on both embryonic and adult zebrafish from infected and control groups. First, blood vessels, branchial regions and livers of control and infected embryos and juveniles were compared (Phelan *et al.*, 2005b). Histopathology included high numbers of monocytes in the blood vessels of the perianal region, marked absence of erythrocytes, high numbers of mucus cells in the buccopharyngeal epithelium and necrotic tissue in the pharyngeal epithelium itself. In addition, pigment cells of infected fish appeared to be irregular in shape compared with the pigment cells of control fish. The lumen of the swim bladder was congested with cell debris in infected fish, and the livers of infected fish also contained necrotic cells. The liver tissue also displayed cytoplasmic vacuolization and pyknosis of hepatocytic nuclei, indicating toxic conditions. Lastly, the hepatic ducts were obstructed in the infected fish but not in the control fish.

Histopathology of adult zebrafish infected with SHRV by intraperitoneal injection was also observed. These findings were in agreement with the gross pathology observed in adults described above. Fig. 22.1 shows comparisons of the scales and epidermis (Fig. 22.1A,B) and ovaries (Fig. 22.1C–E) of control and SHRV-infected adult zebrafish (Phelan *et al.*, 2005b). The infected adult fish presented subepidermal petechial haemorrhages and oedema near the site of injection (Fig. 22.1B), compared with PBS-injected control fish, which showed no signs of infection or inflammation. Females infected with SHRV displayed degeneration of secondary oocytes (Fig. 22.1D,E), such as disruption of the yolks of the secondary oocytes and subsequent reabsorption of the yolk by neighbouring epithelial granulose cells. Further, infected fish also exhibited fluid and inflammatory cell accumulation in the abdominal cavity.

Histopathology associated with infection by other Novirhabdoviruses is similar. In hirame (HIRRV), histological analysis shows necrotic changes in the kidney and spleen (Oseko, 1992; Roberts, 2012). Externally, hirame present with abdominal distension. In fish afflicted with VHSV (e.g. rainbow trout), necrosis of haematopoietic tissue is observed, as well as the presence of degranulated macrophages (Roberts, 2012). Further, fish infected with the North American strain of VHSV also displayed histological evidence of necrosis in the kidney and pancreas (Meyers *et al.*, 1999). Clinical signs of infection of rainbow trout infected with IHNV also include abdominal distension, abnormal behaviour, darkening of the skin and haemorrhage. Histological observations show that necrosis is common in the kidney and spleen (Romero *et al.*, 2005).

Members of the genus *Novirhabdovirus* are distinguished by the presence of a non-virion (NV) gene located between the G and L genes. In most rhabdovirus genomes, over 90% of the nucleotides are protein-coding, making it reasonable to assume that the NV gene must be critical to the virus, but the function remains unknown (Johnson *et al.*, 2000). Studies attempting to characterize a functional role for NV in the pathogenesis of SHRV have led to controversial results. Studies with SHRV have suggested that NV has no critical function in SHRV replication, nor is it required for SHRV pathogenesis (Johnson *et al.*, 2000; Alonso *et al.*, 2004). Others have demonstrated that in IHNV, the NV protein causes cell rounding and

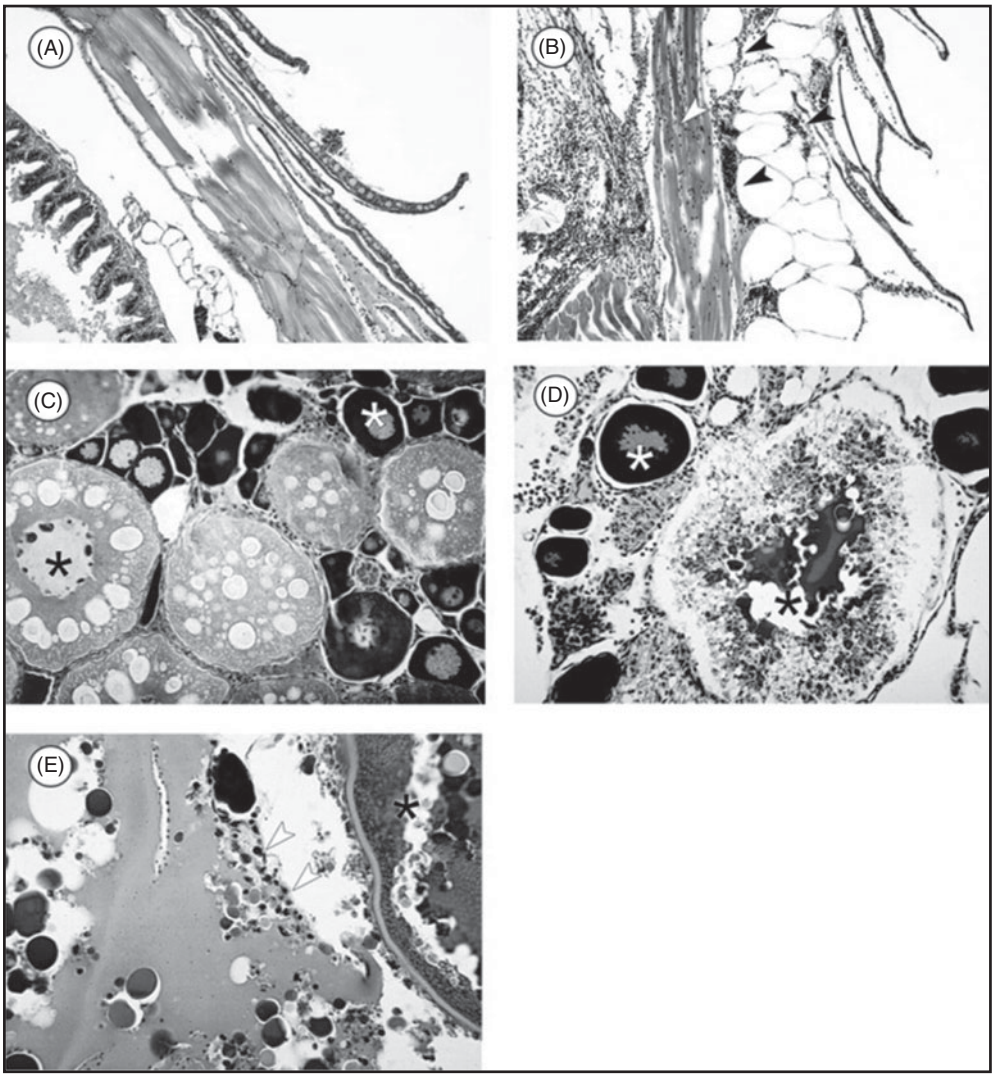

Fig. 22.1. Histopathology of adult zebra fish infected with SHRV by intraperitoneal injection. (A) Normal scales and epidermis of control fish. Magnification 100×. (B) Scales and epidermis of infected fish. Black arrowheads indicate subdermal oedema and haemorrhaging. The white arrowhead indicates haemorrhaging in the underlying muscle tissue. Magnification 100×. (C) Ovaries of control fish showing normal egg development, with generations of ova in different developmental stages. The white asterisk indicates a primary oocyte, the black asterisk indicates a secondary oocyte, and the black arrowhead indicates the epithelial granulosa (nursing) cells. Magnification 200×. (D) Degenerating secondary oocyte of SHRV-infected fish (black asterisk). Primary oocytes seem to be unaffected (white asterisk). Magnification 200×. (E) Epithelial granulosa cells (white arrows) reabsorbing remaining yolk from secondary oocyte. (Previously published in Phelan *et al*. (2005).)

therefore interacts with host cytoskeletal elements (Chiou *et al*., 2000). Recent studies have indicated roles for NV using two separate *Novirhabdoviruses*. One study using recombinant IHNV lacking the NV gene found that NV may function to limit the host interferon (IFN) response in fish because the recombinant virus led to greater transcription of the rainbow trout Mx and IFN1 *in vitro* and had higher levels

of functional IFN (Choi *et al.*, 2011). Recently, studies have also demonstrated that the NV protein of VHSV delays the onset of apoptosis in cell culture, as recombinant VHSV lacking NV induced apoptosis sooner than WT VHSV (Ammayappan and Vakharia, 2011). These reports imply that there are inconclusive results about the role of NV in pathogenesis and whether it is virus-specific.

22.6 Pathogenesis: Immune Response to SHRV

The vertebrate host immune system is comprised of two branches, the innate and adaptive immune systems. The innate and adaptive arms of immunity work synergistically to mount a robust immune response against invading pathogens. Innate immunity is essential for the first line of host response to foreign molecules that have not been previously encountered. It is intrinsic to all eukaryotes and active early in development. Adaptive immunity is restricted to organisms ranging from jawed fish to mammals and gains full competence later in development. The adaptive immune response initiates a specific response against pathogens through recognition of pathogen-specific epitopes. This branch of immunity also provides host immunological memory (Akira, 2009).

The adaptive immune system generates a range of antigen receptors (the T- and B-cell receptors) through DNA rearrangement. Once a pathogen is encountered, lymphocytes with the appropriate antigen receptor to that pathogen eliminate the pathogens in the late stage of infection. All vertebrates possess the components necessary for an adaptive immune response (reviewed in Trede *et al.*, 2004; Guo *et al.*, 2009; Boehm *et al.*, 2012). However, it is known that production of neutralizing antibodies induced by infection or vaccination is important for long-term adaptive immunity to fish rhabdoviruses (Purcell *et al.*, 2012). In addition, fish B cells show phagocytic behaviour (Li *et al.*, 2006), and have a wide range of T-cell associated genes that are upregulated after rhabdoviral infection (Castro *et al.*, 2011). A recent report shows progression towards understanding the fish complement system (Nakao *et al.*, 2011), but the details defining which complement components are critical for virus neutralization remain to be elucidated.

By contrast, innate immunity discriminates self and non-self using germline-encoded receptors called pattern recognition receptors (PRRs). PRRs recognize molecular patterns of components specific to microbial organisms (e.g. single-stranded RNA). Nucleic acids are the primary viral component recognized by the immune system. Protection from rhabdoviral infection depends on innate immunity, in particular the interferon (IFN) system that is rapidly induced upon infection. Teleosts such as zebrafish possess the components critical for mounting an innate and adaptive immune response, similar to that found in other vertebrates.

22.6.1 Innate immune response to SHRV

The innate immune system is the first line of defence against invading organisms. It is composed of cells and molecules that defend the host from infection by pathogens. In contrast to the adaptive branch of immunity, it does not confer protective memory to the host. The innate immune response is also critical for mounting a robust adaptive immune response.

Pathogens are detected by recognition of molecules such as pathogen-associated molecular patterns (PAMPs). Pattern recognition receptors (PRRs) are essential for recognition of PAMPs and generation of immune responses. Activation of signal transduction pathways stimulated by PRR leads to the production of antiviral effector molecules, in particular the type I IFN system.

Recognition of rhabdoviruses in fish is postulated to be similar to that observed in mammals (Zou *et al.*, 2010; Palti, 2011; Purcell *et al.*, 2012). The Toll-like receptors (TLR) are perhaps the best characterized PRRs. They are an evolutionarily-conserved family of PRRs capable of recognizing a

broad range of PAMPs, including viral single- and double-stranded RNA, as well as unmethylated CpG motifs. All TLRs, in vertebrates, possess an extracellular domain containing leucine-rich repeats (LRR), a transmembrane domain and a cytoplasmic Toll/interleukin-1 (IL-1) receptor (TIR) domain (Akira, 2009; Akira and Takeda, 2004). Upon recognition of PAMPs, TLRs recruit adaptor proteins like myeloid differentiation primary response gene (88) (MYD88), MYD88 adaptor-like (MAL), Toll-like receptor adaptor molecule 1 (TICAM1) and TICAM2, and initiate signal transduction pathways (Kumar *et al.*, 2009) (Fig. 22.2A) that mediate inflammatory responses. Note that MAL is also known as toll-interleukin 1 receptor (TIR) domain-containing adaptor protein (TIRAP), TICAM1 is also known as TIR domain-containing adaptor-inducing interferon-β (TRIF) and TICAM2 is also known as TRIF-related adaptor molecule (TRAM). In mammals, TLR3, TLR4, TLR7, TLR8 and TLR9 can initiate antiviral immune responses. TLR3 recognizes double-stranded RNA and uses a MYD88-independent, TICAM1-mediated pathway to trigger an antiviral response. TLR4 has been shown to be activated by several viruses, including respiratory syncytial virus (RSV) (Kurt-Jones *et al.*, 2000) and herpes simplex virus-1 (HSV) (Villalba *et al.*, 2012). It uses both MYD88-dependent and -independent pathways to initiate immune responses. The principle agonists of TLR7 and TLR8 are single-stranded RNA, while TLR9 recognizes unmethylated CpG oligonucleotide (ODN) sequences. All three of these mammalian TLRs use MYD88-dependent pathways to mount antiviral responses.

Fish also possess Tlr proteins that mediate potent immune responses; however, after multiple rounds of genome duplication many of the *tlr* genes that are present likely recognize alternative PAMPs (Sullivan *et al.*, 2009). For example, a thorough examination of gene histories indicates that zebrafish possess *tlr4* genes that are paralogous to mammalian *TLR4* genes (Sullivan *et al.*, 2009). Additionally, zebrafish appear to lack genes homologous to mammalian *CD14* and *MD2*. When exposed to the mammalian TLR4 ligand LPS, Tlr4a and Tlr4b proteins appear unresponsive, failing to activate an NF-κB reporter. Further, fish appear to lack a *TICAM2* ortholog, which is critical for mediating a MYD88-independent, TICAM1-dependent antiviral response in mammals (Sullivan *et al.*, 2007). The most parsimonious explanation is that *ticam2* was lost in teleosts following the divergence of the rayfin and lobefin fishes 450 million years ago. The zebrafish Ticam1 protein has evolved unique structural features that have had important functional impacts on signaling. For example, zebrafish Ticam1 protein, unlike mammalian TICAM1, does not recognize Traf6, due to the lack of a functional Traf6 binding motif. Ticam1-mediated NF-κB activation is triggered through its interaction with Rip1. Additionally, Ticam1 triggers an antiviral interferon response through an Irf3/7-independent mechanism. Zebrafish Ticam1 can interact with Tlr3, which appears to have retained its function through evolution. When Tlr3 is overexpressed, it triggers an NF-κB-dependent immune response (Phelan *et al.*, 2005a). When zebrafish are exposed to SHRV, Tlr3 expression is upregulated. In fugu, Tlr3 is responsive to the dsRNA analogue poly I:C, triggering the activation of an interferon luciferase reporter construct (Matsuo *et al.*, 2008). A novel Tlr22 protein was also discovered in fugu and found to be even more responsive to poly I:C stimulation. Unlike Tlr3, which exhibits restricted expression in endosomes, Tlr22 is expressed on the cell surface. Together, Tlr3 and Tlr22 are thought to provide a more comprehensive defence to viral infection through their combined recognition of dsRNA. In addition to poly I:C stimulation, fish have shown responsiveness to ligands shown to activate other mammalian TLRs. For example, Atlantic salmon can elicit antiviral responses to mammalian TLR7 ligand imidazoquinoline S-27609 (Kileng *et al.*, 2008).

It is important to note that conclusive evidence needs to be obtained to establish direct relationships between fish Tlr proteins and known mammalian agonists. Despite considerable sequence homology, gene histories must be investigated and functional

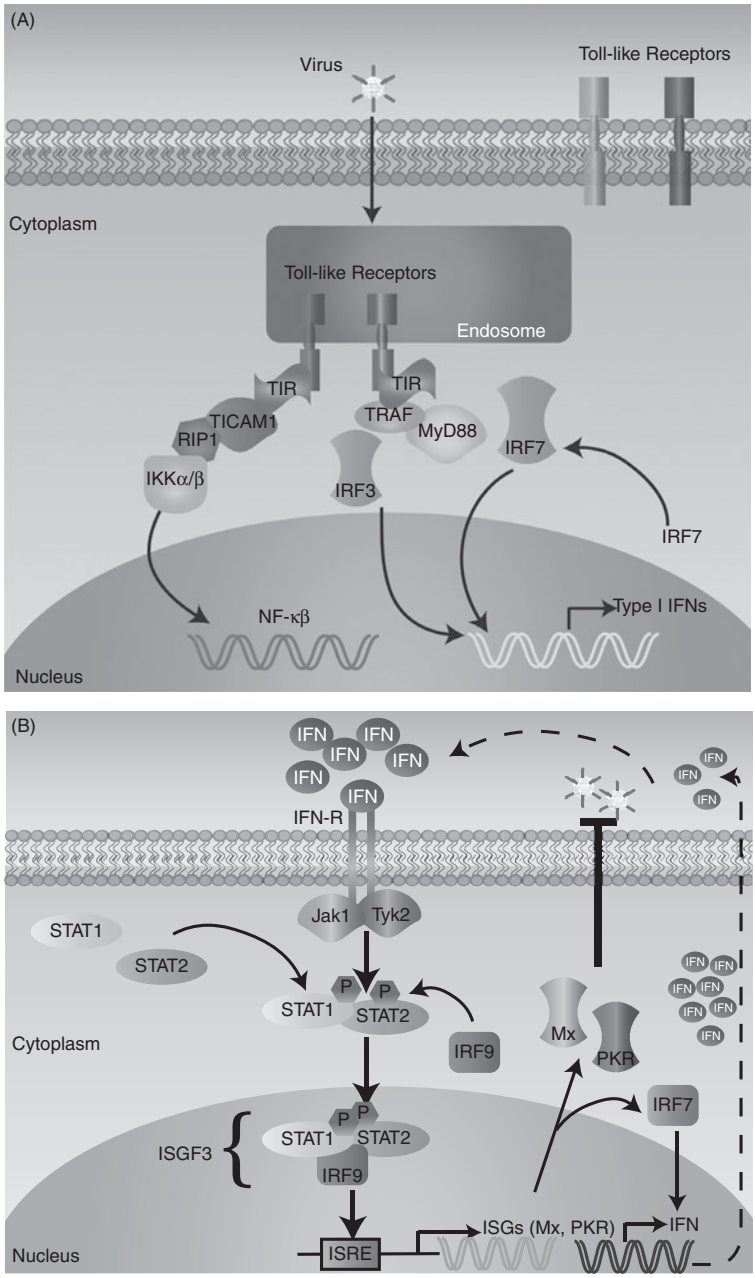

Fig. 22.2. Antiviral signal transduction pathways: Pattern recognition and the antiviral state. (A) Schematic of the mammalian TLR signaling pathway. TLRs are activated once the appropriate ligands are bound and subsequently initiate signaling. Activated NF-κβ will initiate transcription of inflammatory cytokines. When IRF3 and IRF7 are phosphorylated, they are translocated to the nucleus and initiate transcription of IFN. IRF7 contributes to a positive feedback loop for further IFN production. (B) Schematic of the mammalian IFN-R signaling pathway. After IFN is produced from the TLR pathway activation, IFN binds to the IFN-R and activates the expression of ISGs via the JAK-STAT pathway. IRF7 amplifies the IFN response by inducing further expression of IFN. Mx and PKR are examples of IFN-induced proteins with antiviral activity. Note that not all known components are shown in these diagrams. Fish TLR and IFN-R pathways are thought to signal in a similar manner.

assays must be performed in order to prove conservation of Tlr function. The zebrafish model system offers opportunities to bridge the gap between human health and fisheries health because of the wealth of tools and information that are available. Using the zebrafish system, it is possible to interrogate a fish genome to explore questions of the gene history and then perform intensive assays aimed at answering important biological questions that can broadly expand our understanding of fish health (Sullivan *et al.*, 2007, 2009; Sullivan and Kim, 2008).

22.6.2 Innate immune response: IFN and IFN-stimulated genes

During viral infection, critical immune mediators that become activated as a result of PAMP recognition by TLRs are antiviral cytokines such as IFN, and IFN-stimulated genes (ISGs) with antiviral properties (Fig. 22.2B). IFNs mediate the early response to viruses and are crucial for the establishment of the antiviral state. Activation of type I IFN is important in controlling rhabdoviruses, as demonstrated by findings that neither rabies virus nor vesicular stomatitis virus can replicate in cells with activated type I IFN (Purcell *et al.*, 2012). The IFN receptor (IFN-R) pathway signals through the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway, which results in upregulation of more than 300 ISGs, which determine the antiviral state of the host. Many ISGs, such as Mx, have direct antiviral function.

Fish rhabdoviral infections result in a rapid IFN and ISG response (Purcell *et al.*, 2012). Our laboratory was the first to clone full-length zebrafish type I IFN (zfIFN) (Altmann *et al.*, 2003). We demonstrated that zfIFN was able to activate the IFN-inducible Mx promoter. A protective effect was observed when zebrafish cells were transfected with zfIFN and challenged with a fish rhabdovirus. Further, we reported the cloning and characterization of an Mx gene and its corresponding promoter (Altmann *et al.*, 2004). Zebrafish liver cells produced high levels of Mx mRNA in response to induction by a known IFN-inducer, poly I:C (Altmann *et al.*, 2004). Recently, zebrafish IFNs have been characterized in more detail. They are called IFN-psi (Ψ), and are split into two major groups based on their affinity for binding specific receptors (Levraud *et al.*, 2007; Aggad *et al.*, 2009). The IFN response is critical for establishing the antiviral state. IFNs halt protein synthesis in the host cell, thus inhibiting virus replication. A detailed understanding of how IFNs function is critical for development of antiviral therapies. Our laboratory has used SHRV to induce an immune response in zebrafish and has elucidated this signal transduction pathway at the molecular level (Gabor *et al.*, 2013).

22.6.3 Pathogenesis: generation of recombinant SHRV

Researchers have previously reported recovery of infectious recombinant SHRV from a full-length cDNA clone of the viral genome, demonstrating that mutation of the NV-gene had no effect on virus assembly or virulence (Johnson *et al.*, 2000; Alonso *et al.*, 2004). Johnson *et al.* (2000) used reverse genetics to insert a point mutation into the open reading frame (ORF) of the NV-gene (Johnson *et al.*, 2000). This mutation had no effect on viral assembly or pathogenesis in epithelioma papulusom cyprini (EPC) cells. Alonso *et al.* (2004) employed a similar technique, but produced a viable recombinant SHRV with full deletion of NV (Alonso *et al.*, 2004). This virus was produced at the same rate and concentration as wild-type virus in both cultured fish cells and zebrafish, indicating that the NV protein plays no apparent role in fish pathogenesis (Alonso *et al.*, 2004).

Similarly, we have used reverse genetics to express the sequence of green fluorescent protein (GFP) within the NV coding region and successfully recovered GFP-SHRV. Fluorescently labelled copies of either the G-gene or N-gene products were inserted into the NV location of the SHRV cDNA genome containing an NV-deletion. Infectious virus has been recovered, and expression of GFP-SHRV

confirmed by confocal microscopy in cell culture (Fig. 22.3A) and in zebrafish embryos (Fig. 22.3B). SHRV expressing red fluorescent protein (RFP) has also been generated (data not shown). This has enabled us to study viral pathogenesis of SHRV *in vivo*, as well as to assess the immune response to SHRV. Additionally, a number of transgenic zebrafish exist that have fluorescently labelled immune cells (Mathias *et al.*, 2006; Renshaw *et al.*, 2006; Hall *et al.*, 2007, 2009). The use of GFP- or RFP-labelled SHRV, combined with a labelled macrophage cell (Hall *et al.*, 2007), would, for instance, enable visualization of the host immune response to virus infection *in vivo*.

22.7 Using Zebrafish to Model Innate Immunity to SHRV Infection

Understanding how SHRV interacts with the host immune system may provide insights into immune signal transduction pathways as well as antiviral evasion strategies employed by rhabdoviruses. Although SHRV was initially isolated as a potential aetiological agent of EUS, this is likely not the case. However, a zebrafish model for SHRV infection has previously been characterized in our laboratory (Phelan *et al.*, 2005b). Observations were made of pathological effects and clinical disease, as discussed above. In addition, we used this infection model to study the immune response to virus infection. Overall, the zebrafish immune system exhibits a remarkable similarity to that of humans. The zebrafish has been established as a viable model for viral infection and immunity because researchers can observe innate immunity exclusively in embryos, the transitional immune response as the adaptive immune system develops, and the complexity of innate and adaptive immunity in adult fish (Sullivan and Kim, 2008).

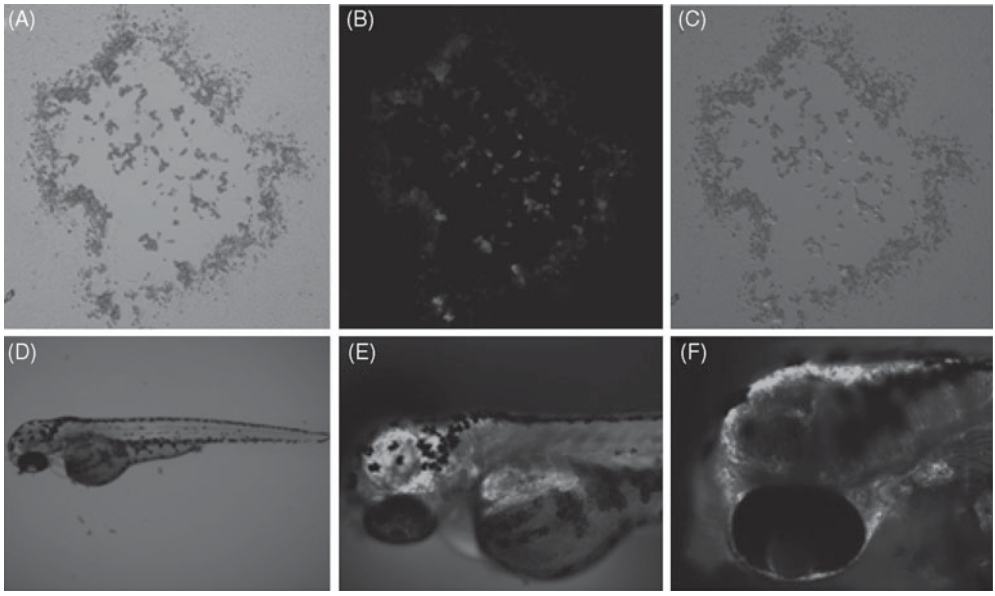

Fig. 22.3. GFP-labelled SHRV generates plaques in culture and causes infection in zebrafish embryos. Reverse genetics were used to generate GFP-labelled SHRV. (A–C) EPC cells in culture were infected with GFP-labelled SHRV. Plaques formed after 24 hours and could be visualized by brightfield (A) or fluorescence (B) microscopy. Brightfield and fluorescence images were merged (C) to demonstrate that the edges of the plaque were green. For all images 10× magnification was used. (D–F) Zebrafish embryos were injected at 48 hours post fertilization with GFP-SHRV to cause a systemic infection. After 24 hours, green fluorescence was clearly observed throughout the zebrafish. Magnification is 4× (D), 10× (E) and 20× (F).

22.8 Diagnosis of SHRV

Although SHRV has never been identified as the sole aetiological agent in a microbial infection, it has been associated with EUS as a potential contributor to this multi-microbe disease. Even so, SHRV has been a useful research tool for laboratory infections of zebrafish and has been used as a model for infection and immunity studies (Altmann *et al.*, 2003, 2004; Phelan *et al.*, 2005b; Sullivan and Kim, 2008). SHRV is a plaque-forming virus that generates small (1–2 mm diameter), uniformly shaped plaques in culture. Confirmation of an SHRV infection in fish tissue may be carried out by incubating supernatants from filtered tissue homogenates with cells in culture. SHRV replicates optimally in epithelioma papulosum cyprinid (EPC) cells at 28°C (Phelan *et al.*, 2005b). Cytopathic effects (CPE) are observed in EPC cells after seven days and are characterized by cell rounding and destruction of the cell monolayer. Infection can also be confirmed by PCR, qRT-PCR or sequence analysis.

A variety of antibodies have been developed against SHRV. Polyclonal antibodies were produced in rabbits, but rabbit anti-SHRV sera had low titres and were toxic to cells. Rabbit antisera also exhibited high levels of background fluorescence and non-specific binding in immunofluorescence assays. Monoclonal antibodies (MAbs) described by Kasornchandra *et al.* (1992b) were developed to improve methods for identification of SHRV. One neutralizing group and one non-neutralizing group of MAb were characterized. The G protein of SHRV was able to induce the production of neutralizing antibody, but not all of the MAbs specific for the G protein neutralized the virus. In immunoprecipitation assays the N protein of SHRV displayed two bands when reacted with MAbs. Since the MAbs each recognized different viral proteins, distinct immunofluorescence staining patterns were produced. MAbs developed against SHRV should prove to be useful tools in immunodiagnostic assays.

None of the antibodies that have been developed to date appear to be optimal for SHRV detection. To our knowledge these MAbs are not commercially available and 20 years have passed since their development. No further use of these Mabs has been reported, and so their efficacy for immunodiagnostics remains unknown. Since SHRV can be detected readily by PCR or qRT-PCR, these techniques may be the best methods to diagnose viral infection. Another possibility is to utilize the GFP-SHRV (see also section 22.6.3) developed in our laboratory to visually observe the infection. Although diagnosis of SHRV is possible, it is not performed routinely, and new diagnostic tools have not been developed for this pathogen.

22.9 Concluding Remarks

SHRV has not been identified as the sole causative agent of any single viral infection in fish, though it is now thought to be associated with multi-microbial infection. This virus has, however, been essential for the development of a rhabdoviral infection model. The use of the zebrafish as a host for SHRV has resulted in a highly effective model for host antiviral immunity. This model has the potential for broad impact on our understanding of viral pathogenesis and immune response in both human health and economically important fisheries. Ultimately, we hope to find new ways to modulate immune function to enhance host response to infection and to develop improved methods for disease prevention and vaccination. If histopathology suggests SHRV infection, this is best diagnosed using PCR and sequencing techniques, or visually using GFP-SHRV. SHRV may be more prevalent in the wild than is currently assumed. SHRV-specific PCR primers could be included in future diagnostic screens to determine if this assumption is correct. Antibody detection of SHRV is possible, but the antibodies described to date are not optimal and are not commercially available. The vast body of scientific information and the reagents that are

becoming readily available in the zebrafish system will provide powerful tools in this effort. The unique properties of the SHRV virus will help us to gain insight into the complex interaction between host and pathogen and this new understanding will further enhance our ability to combat viral disease.

References

Aggad, D., Mazel, M., Boudinot, P., Mogensen, K.E., Hamming, O.J., Hartmann, R., Kotenko, S., Herbomel, P., Lutfalla, G. and Levraud, J.P. (2009) The two groups of zebrafish virus-induced interferons signal via distinct receptors with specific and shared chains. *Journal of Immunology* 183, 3924–3931.

Ahne, W.J., Jørgensen, P.E.V., Olessen, N.J. and Wattanavijarn, W. (1988) Serological examination of a rhabdovirus isolated from snakehead (*Ophicephalus striatus*) in Thailand with ulcerative syndrome. *Journal of Applied Ichthyology* 4, 194–196.

Akira, S. (2009) Innate immunity to pathogens: diversity in receptors for microbial recognition. *Immunological Reviews* 227, 5–8.

Akira, S. and Takeda, K. (2004) Toll-like receptor signalling. *Nature Reviews Immunology* 4, 499–511.

Alonso, M., Kim, C.H., Johnson, M.C., Pressley, M. and Leong, J.A. (2004) The NV gene of snakehead rhabdovirus (SHRV) is not required for pathogenesis, and a heterologous glycoprotein can be incorporated into the SHRV envelope. *Journal of Virology* 78, 5875–5882.

Altmann, S.M., Mellon, M.T., Distel, D.L. and Kim, C.H. (2003) Molecular and functional analysis of an interferon gene from the zebrafish, *Danio rerio*. *Journal of Virology* 77, 1992–2002.

Altmann, S.M., Mellon, M.T., Johnson, M.C., Paw, B.H., Trede, N.S., Zon, L.I. and Kim, C.H. (2004) Cloning and characterization of an Mx gene and its corresponding promoter from the zebrafish, *Danio rerio*. *Developmental & Comparative Immunology* 28, 295–306.

Ammayappan, A. and Vakharia, V.N. (2011) Nonvirion protein of novirhabdovirus suppresses apoptosis at the early stage of virus infection. *Journal of Virology* 85, 8393–8402.

Baldock, F.C., Blazer, V., Callinan, R.B., Hatal, K., Karunasagar, I., Mohan, C.V. and Bondad-Reantaso, M.G. (2005) Outcomes of a short expert consultation on epizootic ulcerative syndrome (EUS): re-examination of causal factors, case definition, and nomenclature. In: Walker, P., Lester, R. and Bondad-Reantaso, M.G. (eds) *Diseases in Aquaculture V*. Asian Fisheries Society, Manila.

Boehm, T., Iwanami, N. and Hess, I. (2012) Evolution of the immune system in the lower vertebrates. *Annual Review of Genomics & Human Genetics* 13, 127–149.

Cann, A.J. (1993) *Principles of Molecular Virology*. Academic Press, San Diego, California.

Castro, R., Bernard, D., Lefranc, M.P., Six, A., Benmansour, A. and Boudinot, R. (2011) T cell diversity and TcR repertoires in teleost fish. *Fish & Shellfish Immunology* 31, 644–654.

Chiou, P.P., Kim, C.H., Ormonde, P. and Leong, J.A. (2000) Infectious hematopoietic necrosis virus matrix protein inhibits host-directed gene expression and induces morphological changes of apoptosis in cell cultures. *Journal of Virology* 74, 7619–7627.

Choi, M.K., Moon, C.H., Ko, M.S., Lee, U.H., Cho, W.J., Cha, S.J., Do, J.W., Heo, G.J., Jeong, S.G., Hahm, Y.S., Harmache, A., Bremont, M., Kurath, G. and Park, J.W. (2011) A nuclear localization of the infectious haematopoietic necrosis virus NV protein is necessary for optimal viral growth. *PLoS One* 6.

Encinas, P., Rodriguez-Milla, M.A., Novoa, B., Estepa, A., Figueras, A. and Coll, J. (2010) Zebrafish fin immune responses during high mortality infections with viral haemorrhagic septicemia rhabdovirus. A proteomic and transcriptomic approach. *BMC Genomics* 11, 518.

Frerichs, G.N. (1995) Viruses associated with the epizootic ulcerative syndrome (EUS) of fish in South-East Asia. *Veterinary Research* 26, 449–454.

Frerichs, G.N., Millar, S.D. and Roberts, R.J. (1986) Ulcerative rhabdovirus in fish in South-East Asia. *Nature* 322, 216.

Frerichs, G.N., Hill, B.J. and Way, K. (1989) Ulcerative disease rhabdovirus – cell-line susceptibility and serological comparison with other fish rhabdoviruses. *Journal of Fish Diseases* 12, 51–56.

Gabor, K.A., Stevens, C.R., Pietraszewski, M.J., Gould, T.J., Shim, J., Yodes, J.A., Lam, S.H., Gong, Z., Hess, S.T. and Kim, C.H. (2013) Super resolution microscopy reveals that caveolin-1 is required for spatial organization of CRFB1 and subsequent antiviral signaling in zebrafish. PLoS ONE 8, e68759.

Guo, P., Hirano, M., Herrin, B.R., Li, J., Yu, C., Sadlonova, A. and Cooper, M.D. (2009) Dual nature of the adaptive immune system in lampreys. *Nature* 459, 796–801.

Hall, C., Flores, M.V., Storm, T., Crosier, K. and Crosier, P. (2007) The zebrafish lysozyme C promoter drives myeloid-specific expression in transgenic fish. *BMC Developmental Biology* 7, 42.

Hall, C., Flores, M.V., Crosier, K. and Crosier, P. (2009) Live cell imaging of zebrafish leukocytes. *Methods in Molecular and Cellular Biology* 546, 255–271.

Hedrick, R.P., Eaton, W.D., Fryer, J.L., Groberg, W.G. Jr and Boonyaratapalin, S. (1986) Characteristics of a birnavirus isolated from cultured sand goby (*Oxyeleotris marmoratus*). *Diseases of Aquatic Organisms* 1, 219–225.

Hermann, A.C., Millard, P.J., Blake, S.L. and Kim, C.H. (2004) Development of a respiratory burst assay using zebrafish kidneys and embryos. *Journal of Immunological Methods* 292, 119–129.

Hoffman, B., Beer, M., Schutze, H. and Mettenleiter, T. (2005) *Fish Rhabdoviruses: Molecular Epidemiology and Evolution*, Springer, Berlin.

Johansson, T., Ostman-Myllyoja, L., Hellstrom, A., Martelius, S., Olesen, N.J. and Bjorklund, H. (2002) A novel fish rhabdovirus from Sweden is closely related to the Finnish rhabdovirus 903/87. *Virus Genes* 25, 127–138.

John, K.R., George, M.R., Richards, R.H. and Frerichs, G.N. (2001) Characteristics of a new reovirus isolated from epizootic ulcerative syndrome infected snakehead fish. *Diseases of Aquatic Organisms* 46, 83–92.

Johnson, M.C., Simon, B.E., Kim, C.H. and Leong, J.A. (2000) Production of recombinant snakehead rhabdovirus: the NV protein is not required for viral replication. *Journal of Virology* 74, 2343–2350.

Kasornchandra, J., Engelking, H.M., Lannan, C.N., Rohovec, J.S. and Fryer, J.L. (1992a) Characteristics of 3 rhabdoviruses from snakehead fish *Ophicephalus striatus*. *Diseases of Aquatic Organisms* 13, 89–94.

Kasornchandra, J., Caswell-Reno, P., Lannan, C.N. and Fryer, J.L. (1992b) Development and characterization of monoclonal antibodies against the snakehead rhabdovirus. *Journal of Aquatic Animal Health* 4, 286–291.

Kileng, O., Albuquerque, A. and Robertsen, B. (2008) Induction of interferon system genes in Atlantic salmon by the imidazoquinoline S-27609, a ligand for Toll-like receptor 7. *Fish & Shellfish Immunology* 24, 514–522.

Kim, D.H., Oh, H.K., Eou, J.I., Seo, H.J., Kim, S.K., Oh, M.J., Nam, S.W. and Choi, T.J. (2005) Complete nucleotide sequence of the hirame rhabdovirus, a pathogen of marine fish. *Virus Research* 107, 1–9.

Kumar, H., Kawai, T. and Akira, S. (2009) Pathogen recognition in the innate immune response. *Biochemical Journal* 420, 1–16.

Kurt-Jones, E.A., Popova, L., Kwinn, L., Haynes, L.M., Jones, L.P., Tripp, R.A., Walsh, E.E., Freeman, M.W., Golenbock, D.T., Anderson, L.J. and Finberg, R.W. (2000) Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nature Immunology* 1, 398–401.

Levraud, J.P., Boudinot, P., Colin, I., Benmansour, A., Peyrieras, N., Herbomel, P. and Lutfalla, G. (2007) Identification of the zebrafish IFN receptor: implications for the origin of the vertebrate IFN system. *Journal of Immunology* 178, 4385–4394.

Li, J., Barreda, D.R., Zhang, Y.A., Boshra, H., Gelman, A.E., Lapatra, S., Tort, L. and Sunyer, J.O. (2006) B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities. *Nature Immunology* 7, 1116–1124.

Lilley, J.H. and Frerichs, G.N. (1994) Comparison of rhabdoviruses associated with epizootic ulcerative syndrome (EUS) with respect to their structural proteins, cytopathology, and serology. *Journal of Fish Diseases* 17, 513–522.

Lio-Po, G.D., Traxler, G.S., Albright, L.J. and Leano, E.M. (2000) Characterization of a virus obtained from snakeheads *Ophicephalus striatus* with epizootic ulcerative syndrome (EUS) in the Philippines. *Diseases of Aquatic Organisms* 43, 191–198.

Lopez-Munoz, A., Roca, F.J., Sepulcre, M.P., Meseguer, J. and Mulero, V. (2010) Zebrafish larvae are unable to mount a protective antiviral response against waterborne infection by spring viremia of carp virus. *Developmental & Comparative Immunology* 34, 546–552.

Mathias, J.R., Perrin, B.J., Liu, T.X., Kanki, J., Look, A.T. and Huttenlocher, A. (2006) Resolution of inflammation by retrograde chemotaxis of neutrophils in transgenic zebrafish. *Journal of Leukocyte Biology* 80, 1281–1288.

Matsuo, A., Oshiumi, H., Tsujita, T., Mitani, H., Kasai, H., Yoshimizu, M., Matsumoto, M. and Seya, T. (2008) Teleost TLR22 recognizes RNA duplex to induce IFN and protect cells from birnaviruses. *Journal of Immunology* 181, 3474–3485.

Meyers, T.R., Short, S. and Lipson, K. (1999) Isolation of the North American strain of viral hemorrhagic septicemia virus (VHSV) associated with epizootic mortality in two new host species of Alaskan marine fish. *Diseases of Aquatic Organisms* 38, 81–86.

Mork, C., Hershberger, P., Kocan, R., Batts, W. and Winton, J. (2004) Isolation and characterization of a rhabdovirus from starry flounder (*Platichthys stellatus*) collected from the northern portion of Puget Sound, Washington, USA. *Journal of General Virology* 85, 495–505.

Nakao, M., Tsujikura, M., Ichiki, S., Vo, T.K. and Somamoto, T. (2011) The complement system in teleost fish: progress of post-homolog-hunting researches. *Developmental & Comparative Immunology* 35, 1296–1308.

Nayak, A.S., Lage, C.R. and Kim, C.H. (2007) Effects of low concentrations of arsenic on the innate immune system of the zebrafish (*Danio rerio*). *Toxicological Sciences* 98, 118–124.

Nishizawa, T., Yoshimizu, M., Winton, J., Ahne, W. and Kimura, T. (1991a) Characterization of structural proteins of hirame rhabdovirus, HRV. *Diseases of Aquatic Organisms* 10, 167–172.

Nishizawa, T., Yoshimizu, M., Winton, J.R. and Kimura, T. (1991b) Comparison of genome size and synthesis of structural proteins of hirame rhabdovirus, infectious hematopoietic necrosis virus, and viral hemorrhagic septicemia virus. *Fish Pathology* 26, 77–81.

Novoa, B., Romero, A., Mulero, V., Rodriguez, I., Fernandez, I. and Figueras, A. (2006) Zebrafish (*Danio rerio*) as a model for the study of vaccination against viral haemorrhagic septicemia virus (VHSV). *Vaccine* 24, 5806–5816.

Oidtmann, B. (2012) Review of biological factors relevant to import risk assessments for epizootic ulcerative syndrome (*Aphanomyces invadans*). *Transboundary Emerging Diseases* 59, 26–39.

Oseko, N., Yoshimizu, M. and Kimura, T. (1992) Pathogenicity of Rhabdovirus olivaceus (hirame rhabdovirus; HRV) for salmonid fish. In: Kimura, T. (ed.) *Salmonid Diseases: Proceedings of the Oji International Symposium on Salmonid Diseases*. Hokkaido University Press, Hokkaido, Japan.

Palti, Y. (2011) Toll-like receptors in bony fish: from genomics to function. *Developmental and Comparative Immunology* 35, 1263–1272.

Phelan, P.E., Mellon, M.T. and Kim, C.H. (2005a) Functional characterization of full-length TLR3, IRAK-4, and TRAF6 in zebrafish (*Danio rerio*). *Molecular Immunology* 42, 1057–1071.

Phelan, P.E., Pressley, M.E., Witten, P.E., Mellon, M.T., Blake, S. and Kim, C.H. (2005b) Characterization of snakehead rhabdovirus infection in zebrafish (*Danio rerio*). *Journal of Virology* 79, 1842–1852.

Purcell, M.K., Laing, K.J. and Winton, J.R. (2012) Immunity to fish rhabdoviruses. *Viruses* 4, 140–166.

Renshaw, S.A., Loynes, C.A., Trushell, D.M., Elworthy, S., Ingham, P.W. and Whyte, M.K. (2006) A transgenic zebrafish model of neutrophilic inflammation. *Blood* 108, 3976–3978.

Rieder, M. and Conzelmann, K.K. (2011) Interferon in rabies virus infection. *Advances in Virus Research* 79, 91–114.

Roberts, R.J. (2012) *Fish Pathology*. Wiley-Blackwell, Oxford.

Roberts, R.J., Frerichs, G.N., Tonguthai, K. and Chinabut, S. (1994) Epizootic ulcerative syndrome of farmed and wild fishes. In: Muir, J.F. and Roberts, R.J. (eds) *Recent Advances in Aquaculture V*. Blackwell Science, Oxford.

Romero, A., Figueras, A., Tafalla, C., Thoulouze, M.I., Bremont, M. and Novoa, B. (2005) Histological, serological and virulence studies on rainbow trout experimentally infected with recombinant infectious hematopoietic necrosis viruses. *Diseases of Aquatic Organisms* 68, 17–28.

Sanders, G.E., Batts, W.N. and Winton, J.R. (2003) Susceptibility of zebrafish (*Danio rerio*) to a model pathogen, spring viremia of carp virus. *Comparative Medicine* 53, 514–521.

Subramaniam, S., Chew-Lim, M., Chong, S.Y., Howe, J., Ngoh, G.H. and Chan, Y.C. (1993) Molecular and electron microscopic studies of infectious pancreatic necrosis virus from snakehead. *IXth International Congress of Virology*, Glasgow, UK.

Sullivan, C. and Kim, C.H. (2008) Zebrafish as a model for infectious disease and immune function. *Fish & Shellfish Immunology* 25, 341–350.

Sullivan, C., Postlethwait, J.H., Lage, C.R., Millard, P.J. and Kim, C.H. (2007) Evidence for evolving Toll-IL-1 receptor-containing adaptor molecule function in vertebrates. *Journal of Immunology* 178, 4517–4527.

Sullivan, C., Charette, J., Catchen, J., Lage, C.R., Giasson, G., Postlethwait, J.H., Millard, P.J. and Kim, C.H. (2009) The gene history of zebrafish tlr4a and tlr4b is predictive of their divergent functions. *Journal of Immunology* 183, 5896–5908.

Trede, N.S., Langenau, D.M., Traver, D., Look, A.T. and Zon, L.I. (2004) The use of zebrafish to understand immunity. *Immunity* 20, 367–379.

Villalba, M., Hott, M., Martin, C., Aguila, B., Valdivia, S., Quezada, C., Zambrano, A., Concha, M.I. and Otth, C. (2012) Herpes simplex virus type 1 induces simultaneous activation of Toll-like receptors 2 and 4 and expression of the endogenous ligand serum amyloid A in astrocytes. *Medical Microbiology and Immunology* 201, 371–379.

Wang, L., Zhang, H.X., Zhang, J.H., Chen, W.H., Ruan, X.F. and Xia, C. (2006) In vitro effects of recombinant zebrafish IFN on spring viremia of carp virus and infectious hematopoietic necrosis virus. *Journal of Interferon & Cytokine Research* 26, 256–259.

Wattanavijarn, W.T., Tangtronpiros, J. and Wattanodorn, K. (1986) Viruses of ulcerative diseased fish in Thailand, Burma, and Laos. *First International Conference on the Impact of Viral Diseases on the Development of Asian Countries*. Bangkok, Thailand.

Wattanavijarn, W.T., Torchy, C., Tangtrongpiros, J. and De Kinkelin, P. (1988) Isolation of a birnavirus belonging to Sp serotype, from southeast Asia fishes. *Bulletin of the European Association of Fish Pathologists* 8, 106–107.

Zou, J., Bird, S. and Secombes, C. (2010) Antiviral sensing in teleost fish. *Current Pharmaceutical Design* 16, 4185–4193.

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