Current Topics in Microbiology and Immunology

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Swine Influenza

Springer

Current Topics in Microbiology and Immunology

Volume 370

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Responsible Series Editor: Richard W. Compans

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ISSN 0070-217X
ISBN 978-3-642-36870-7 ISBN 978-3-642-36871-4 (eBook)
DOI 10.1007/978-3-642-36871-4
Springer Heidelberg New York Dordrecht London

Library of Congress Control Number: 2013933292

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

From the first detailed clinical description of the disease in the Midwestern United States in 1918, to the isolation of the causative agent, the first of any influenza virus, in 1930 (Shope 1931) to its role in the genesis of the 2009 human pandemic (Garten et al. 2009), swine have played a central role in the ecology of influenza. Although not considered the major natural reservoir for influenza A viruses, that distinction belongs to aquatic waterfowl, swine are host to a limited but dynamic assortment of viruses (Webster et al. 1992). A number of subtypes of influenza A viruses of human and avian origin, including H1, H2, H3, H4, H5, H7, and H9, have been isolated from global swine populations (reviewed in Brockwell-Staats et al. 2009). Most of these isolations have, however, been limited in number and it is only H1 and H3 influenza viruses that are known to have formed stable lineages in swine. In this respect, swine influenza viruses (SIV) are similar to their counterparts in humans where H1 and H3 viruses have also been maintained. The nature of these H1 and H3 viruses differs between the two host populations, however, and, as discussed throughout this book, are even different in swine populations in different geographic regions of the world due to multiple introductions of avian and human influenza viruses.

The dynamic nature of SIV poses difficulties for the swine industry as a recurring respiratory disease, and also for public health as a source of zoonotic infection. Human infections with SIV have been recorded regularly since the introduction of more routine testing in humans. Many of these zoonotic events have occurred in instances where humans and swine are in close contact and have typically been dead-end events with little to no further spread in humans. The virologic features of SIV that limit their spread in humans are largely unknown, but the host range barrier between human and swine highlights the fact that adaptation of a virus in one mammalian host does not necessarily mean that it is well adapted to replication in another (Landolt et al. 2003). This observation is somewhat in conflict with earlier dogmas in influenza where it was suggested that mammalian passage of avian influenza viruses was a prerequisite for the emergence of human pandemics. Swine were often identified as this mammalian host due to a number of factors including the limited number of other described natural mammalian hosts and the

fact that swine appeared unique in having the receptors preferred by both human and avian influenza viruses (Ito et al. 1998). The observation that swine appeared uniquely susceptible to avian and human viruses and that avian viruses grew poorly in humans led to the postulation that these animals were the mixing vessel for human pandemic viruses; and for a number of years popular thinking, without much definitive proof, was that the 1957 and 1968 human pandemics likely arose in pigs (Scholtissek et al. 1978). Subsequent human infections with H5N1, H7, and H9N2 viruses with domestic poultry as the likely source and realizations that swine were not unique in their ability to harbor avian and human viruses shifted thinking toward poultry being as important as swine as reservoirs of viruses with pandemic potential. Indeed, the global spread of highly pathogenic H5N1viruses focused a lot of research effort and funding toward avian hosts at the expense of solidifying activities in swine. Although surveillance and research activities of influenza in swine continued, and to some degree increased, during the first decade of the twenty-first century, these activities were dwarfed by the efforts going on in wild and domestic poultry species. The isolation of a novel influenza virus (i.e., pandemic H1N1) from a 10-year-old boy in California in April 2009 indicated that more of the influx of resources should have been funneled into further understanding the global SIV situation. The virus from the 10-year-old was obviously of SIV ancestry, but it was different enough from any other virus characterized that its direct precursors still remain a mystery. In addition, in 2012 zoonotic transmission of SIV (both H3N2 and H1N2 subtypes) containing the matrix gene from the pandemic H1N1 virus was reported. These strains appeared to be able to spread more easily from pigs to people than other influenza viruses of swine. More than 300 people from 10 states were reported to have been infected with these new strains resulting in hospitalizations and 1 death; limited human-to-human transmission was detected (Lindstrom et al. 2012). Importantly, the main risk factor for infection was exposure to pigs, mostly in the context of agricultural fair settings.

With these events firmly at center stage, it is a good opportunity to review what we know about SIV as a disease of swine and also as a continued zoonotic threat. The 15 chapters presented in this book provide contemporary reviews of research on SIV. The book begins with a general overview of influenza viruses by Stephan Pleschka discussing the virus and its replication in detail. The history of SIV in North America, Europe, and Asia is discussed by Stacey Schultz-Cherry, Christopher Olsen, and Bernard Easterday, by Roland Zell, Christoph Scholtissek, and Stephan Ludwig, and by Huachen Zhu, Richard Webby, Tommy Lam, David Smith, Malik Peiris, and Yi Guan, respectively. As indicated in these reviews, the European, North American, and Asian SIV evolution follows different pathways. Whereas descendants of classical SIV and the novel triple reassortant viruses are found in North America, avian-like swine H1N1 viruses emerged in Europe in 1979 after an avian to swine transmission and spread to all major European pig-producing countries where they circulate with H3N2 and H1N2 reassortants. Classical swine H1N1, human-origin H3N2, avian-like H1N1 and the triple reassortant viruses all co-circulate in Asian pigs. The clinicopathological features of SIV infections in pigs are described by Bruce Janke. Macroscopic and

microscopic lesions of SIV infection, after natural and experimental infection, are described. The use of accurate diagnostics assays for diagnosis and surveillance for SIV are summarized by Susan Detmer, Marie Gramer, Sagar Goyal, Montserrat Torremorell, and Jerry Torrison. Since our collective knowledge regarding the worldwide occurrence of influenza among swine is incomplete, this review focuses on basic laboratory assays needed for the detection of the virus and viral nucleic acids within clinical samples and for antiviral antibodies in serum samples.

The epidemiology of swine influenza worldwide is of exceptional importance with the potential of the pig acting as a "mixing vessel" where both avian and human influenza viruses can undergo genetic reassortment resulting in the creation of novel viruses. The reviews by Alessio Lorusso, Amy Vincent, Marie Gramer, Kelly Lager, and Janice Ciacci-Zanella on North American, by Ian Brown on European, and by Young-Ki Choi, Philippe Noriel Pascua, and Min-Suk Song on Asian swine influenza epidemiology shed light on how this unique ability of pigs results in ever expanding new genotypes and subtypes in pigs. Vaccination is still one of the most important and effective strategies to prevent and control influenza for both the animal and human population. The review by Kristien van Reeth and Wenjun Ma discusses the current and future options to control this economically important swine disease.

The zoonotic aspects of SIV infections are reviewed by Whitney Baker and Gregory Gray. Most of these infections have been sporadic cases with a recent increase of case reports in concert with modern pig farming and the emergence of triple reassortant SIV. The advent of pandemic H1N1 and its impact on human health is discussed by Ian York and Ruben Donis, while Julia Keenliside discusses its impact on animal populations. Hadi Yassine, Chang-Won Lee, and Yehia Saif describe another important interspecies transmission event of influenza A viruses, namely the one between swine and poultry. Swine viruses are continuously isolated from poultry species, especially turkeys, and they are causing economic losses. Finally, Elena Govorkova and Jonathan McCullers cover the critical area of approved and investigational antiviral drugs.

We would like to thank the contributors for their patience during the assembly of this volume. We hope that all readers will gain insight from these contributions that will enhance their individual research and teaching activities.

J. A. Richt
R. J. Webby

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Contents

Overview of Influenza Viruses

Stephan Pleschka

Abstract The influenza virus (IV) is still of great importance as it poses an immanent threat to humans and animals. Among the three IV-types (A, B, and C) influenza A viruses are clinically the most important being responsible for severe epidemics in humans and domestic animals. Aerosol droplets transmit the virus that causes a respiratory disease in humans that can lead to severe pneumonia and ultimately death. The high mutation rate combined with the high replication rate allows the virus to rapidly adapt to changes in the environment. Thereby, IV escape the existing immunity and become resistant to drugs targeting the virus. This causes annual epidemics and demands for new compositions of the yearly vaccines. Furthermore, due to the nature of their segmented genome, IV can recombine segments. This can eventually lead to the generation of a virus with the ability to replicate in humans and with novel antigenic properties that can be the cause of a pandemic outbreak. For its propagation the virus binds to the target cells and enters the cell to replicate its genome. Newly produced viral proteins and genomes are packaged at the cell membrane where progeny virions are released. As all viruses IV depends on cellular functions and factors for their own propagation, and therefore intensively interact with the cells. This dependency opens new possibilities for anti-viral strategies.

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Current Topics in Microbiology and Immunology (2013) 370: 1–20
DOI: 10.1007/82_2012_272

Published Online: 3 November 2012

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1 Introduction

Influenza viruses (IVs) are a continuous and severe global threat to mankind and many animal species. The resulting disease gives rise to thousands of deaths and enormous economic losses in livestock each year. Clearly, influenza is a highly contagious, acute respiratory disease with global significance that affects all age groups and can occur repeatedly. Since waterfowl represents the natural reservoir for the etiological agent of the disease—the influenza and many other animal species can be infected, the virus cannot be eradicated. Therefore, a constant re-emergence of the disease will continue to occur (Palese and Shaw 2007; Webster 1999; Wilschut 2005; Wright et al. 2007). Epidemics appear in the human population almost annually and are due to an antigenic change of the viral surface glycoproteins (Fig. 1). Furthermore, highly pathogenic strains of influenza A virus have emerged unpredictably but repeatedly in recent history as pandemics like the "Spanish-Flu" that caused the death of 20–40 million people worldwide (Taubenberger et al. 2000; Webster 1999). The 2009 pandemic outbreak of the swine-origin IV (S-OIV, "Mexico-Flu") and its rapid spread around the world, as well as repeated human infections with highly pathogenic avian IV (HPAIV) of the H5-subtype demonstrated the imminent danger that IV continues to pose to both the human population and economically relevant animals.

2 The Virus and Its Replication

2.1 The Virion

IVs belong to the family of the *Orthomyxoviridae* and possess a segmented, single-stranded RNA-genome with negative orientation. IVs are divided into three types, A, B, and C based on the genetic and antigenic differences. They infect mammals and birds. Among the three types, influenza A viruses are clinically the most important pathogens and have been responsible for severe epidemics in humans and domestic animals in the past. Thus the focus of this chapter will be on type-A influenza viruses. A detailed description of the viral proteins and the replication

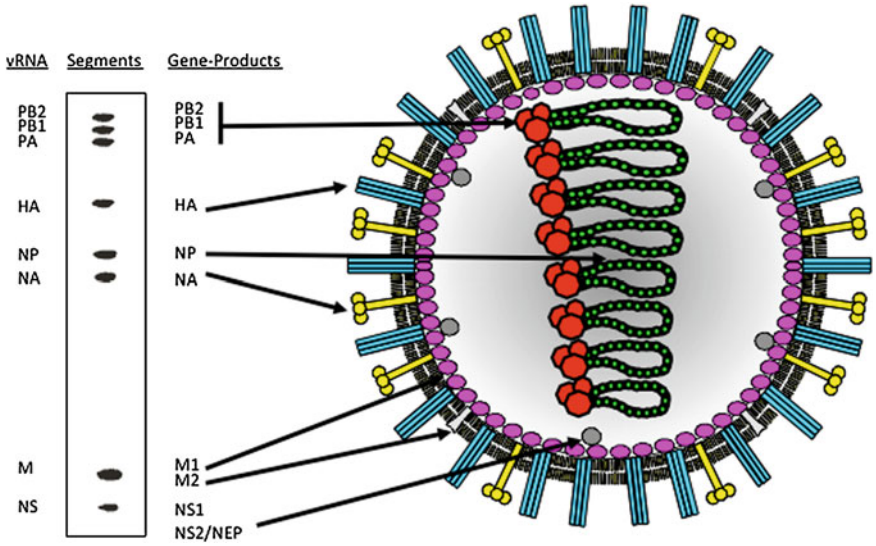

Fig. 1 The influenza A virus particle. Schematic representation of the spherical influenza A virus particle that has a diameter of about 100 nm. The eight viral RNA segments were separated by urea-polyacrylamide gel electrophoresis and visualized by silver staining (*left*). The corresponding gene products and their presumed location in the virus particle are indicated (*right*). PB1-F2 and NS1 are not a structural part of the mature virion. For details see text

cycle of influenza A viruses can be found elsewhere (Ludwig et al. 1999; Palese and Shaw 2007; Wright et al. 2007). Therefore, only an overview on these topics is given without referring to individual references.

The influenza A virus particle is composed of a lipid envelope derived from the host cell and of 9 or 10 structural virus proteins (Fig. 1 and Table 1). The components of the RNA-dependent RNA-polymerase complex (RdRp), PB2, PB1, and PA are associated with the ribonucleoprotein complex (RNP) and are encoded by the vRNA segments 1–3. The PB1 segment of many, but not all, influenza A virus strains also contains a +1-reading frame encoding the recently discovered PB1-F2 protein (Chen et al. 2001).

The viral surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) are expressed from vRNA segments 4 and 6, respectively. The nucleoprotein (NP), the major component of the RNPs, is encoded by segment 5 and associates with the vRNA segments. Each of the two smallest vRNA segments code for two proteins. The matrix protein (M1) is co-linear translated from the mRNA of segment 7 and forms an inner layer within the virion. A spliced version of the mRNA gives rise to a third viral transmembrane component, the M2 protein, which functions as a pH-dependent ion channel. Employing a similar coding strategy, segment 8 harbors the sequence information for the nonstructural NS1 protein and the nuclear export protein (NEP). NEP is a minor component of the virion and is found associated with the M1 protein.

Table 1 Influenza A Virus Genome (strain A/PR/8/34)

Segment	vRNA	Protein	AA	Function(s)
1	2,341	PB2	759	Cap-binding subunit of the viral RdRp; cap-binding
2	2,341	PB1	757	Central location of the polymerase domain of the viral RdRp
		PB1-F2	87–91	Pro-apoptotic activity
3	2,233	PA	716	Cap-snatching endonuclease subunit of the viral RdRp
4	1,778	HA	566	Surface glycoprotein; receptor binding, membrane fusion
5	1,565	NP	498	Nucleoprotein; encapsidation of viral genomic and anti-genomic RNA
6	1,413	NA	454	Surface glycoprotein; receptor destroying Neuraminidase activity
7	1,027	M1	252	Matrixprotein
		M2	97	Ion channel activity, protecting HA conformation
8	890	NS1	230	Regulation of viral RdRp activity Interferon antagonist; Enhancer of viral mRNA translation; inhibition of (i) pre-mRNA splicing, (ii) cellular mRNA-polyadenylation, (iii) PKR activity,
		NEP	121	Nuclear export factor

Table 1 summarizes details of the genome segments, the encoded viral proteins and their respective function.

2.2 The Influenza Virus Replication Cycle: Viral Proteins and Their Function

2.2.1 Adsorption and Entry

The viral replication cycle is initiated by the binding of the HA to sialic-acid (neuraminic acid) containing cellular membrane resident molecules that act as receptors determinants. For example, it was shown that the epidermal growth factor receptor (EGFR) promotes uptake of IV into host cells (Eierhoff et al. 2010). Subsequently, the virus particle is taken up via endocytosis (Fig. 2) [For references: (Palese and Shaw 2007; Wright et al. 2007)]. Due to the different preferences for specific receptor determinants on the target cells of birds and humans, HA is regarded as a possible restriction factor. HAs of avian viruses bind to Sia2-3Gal-terminated sialylglycoconjugates, whereas those of human IV display a Sia2-6Gal-containing receptor-binding specificity [Reviewed in (Paulson 1985) see also (Connor et al. 1994)]. Nevertheless, it was recently shown that a strictly avian H7-type HPAIV carrying the NS segment of a H5-type HPAIV could

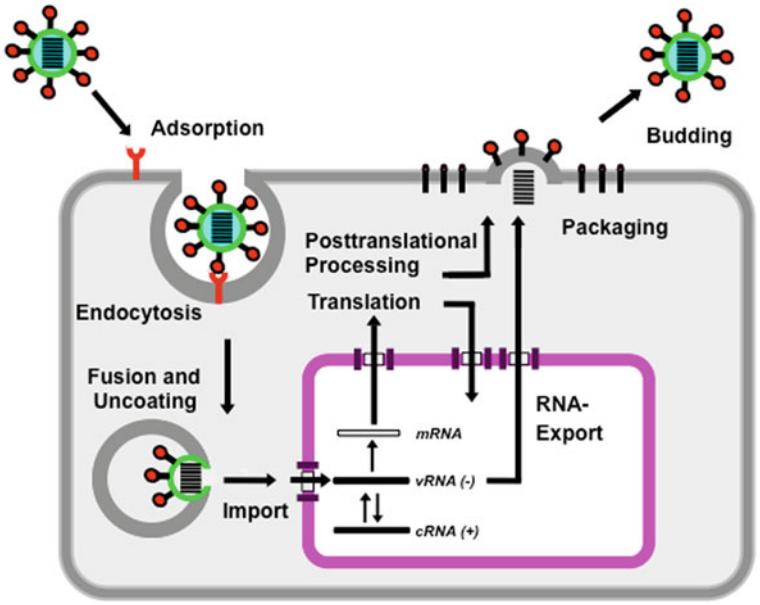

Fig. 2 The influenza viruses replication cycle. The virion attaches to the cellular receptor determinant. The receptor-bound particle enters the cell via endocytosis. After fusion of the viral and the endosomal membrane the viral genome is released into the cytoplasm. The RNPs are transported into the nucleus where replication and transcription of the viral RNA segments occur. The mRNAs are exported into the cytoplasm and are translated into viral proteins. The viral glycoproteins enter the exocytotic transport pathway to the cell surface. Replicative viral proteins enter the nucleus to amplify the viral genome. In the late stage of the infection cycle newly synthesized RNPs are exported from the nucleus and are assembled into progeny virions that bud from the cell surface

acquire the ability to replicate more efficiently in mammalian cell culture, and in contrast to the wild type was able to infect mice causing disease and death (Ma et al. 2010). Furthermore, additional NS reassortants displayed altered propagation ability of the H7-type HPAIV (Wang et al. 2010). Taken together, these results shed further light on the importance of the NS segment for viral replication, molecular pathogenicity and host range, as well as the possible consequences of a reassortment between naturally occurring H7 and H5 type HPAIVs. This indicates that the receptor HA-specificity, although important is not the sole host range and tropism determining factor.

The HA has to undergo a multitude of maturation steps, which are completely dependent on interactions with the protein processing machinery of the infected cell. To gain insight into intra-cellular post-translational processing and transport of glycoproteins, the HA has long been used as a model protein; and HA is probably the best analyzed virus component. A great amount of data has accumulated on the maturation and function of the HA during the viral replication cycle [For overviews: (Ludwig et al. 1999; Palese and Shaw 2007; Wright et al. 2007)].

The HA is a type I glycoprotein. The precursor HA_0 of the mature HA follows the exocytotic cellular transport pathway from the rER via the Golgi complex and the trans-Golgi network (TGN). In polarized epithelial cells, which represent the major viral target cell type in the respiratory and intestinal tracts, HA is transported to the apical surface and thereby defines the site of virus release (Gottlieb et al. 1986; Rindler et al. 1984; Rodriguez-Boulan et al. 1983, 1984). The *N*-terminal signal peptide of the nascent HA is co-translationally recognized by the signal recognition particle (SRP). The complex (SRP/HA/ribosome) binds to the SRP receptor in the ER membrane and the protein chain is transferred into the lumen of the rER (Palese and Shaw 2007). The signal peptide is cleaved off by a luminal signal peptidase. After the translocation into the rER is completed, the HA_0 remains anchored in the rER membrane by a C-terminal hydrophobic sequence. In the rER, the HA_0 becomes *N*-glycosylated. Folding of the HA_0 and intra-molecular disulfide bond formation occurs co- and post-translationally in the rER (Braakman et al. 1991). Folding intermediates of the HA_0 with incomplete disulfide bonds are bound by chaperones. These promote proper folding, oligomeric assembly and quality control of newly synthesized glycoproteins in the ER. The completely folded HA_0 is released from the chaperones only after the remaining glucose is removed (Braakman et al. 1991; Chen et al. 1995; Hebert et al. 1995, 1997; Peterson et al. 1995; Tatu and Helenius 1997). Misfolded HA_0 is degraded in the rER (Copeland et al. 1986; Gething et al. 1986; Hurtley et al. 1989). Properly folded HA_0 monomers assemble into trimers in the rER and are selectively transported to the *cis*-golgi compartment (Ceriotti and Colman 1990).

Another post-translational modification of the HA is acylation of conserved cysteine residues in the cytoplasmatic tail with long-chain fatty acids (Schmidt 1982; Veit et al. 1991). The corresponding acyltransferase is located in the rER (Chen et al. 2001) and the HA_0 is acylated before it reaches the Golgi. The results of several studies suggest that acylation can modulate the fusion activity of the HA (Fischer et al. 1998; Lambrecht and Schmidt 1986; Melikyan et al. 1997; Naeve and Williams 1990; Philipp et al. 1995; Simpson and Lamb 1992; Steinhauer et al. 1991), but has no major effect on the post-translational processing, intra-cellular transport, or receptor binding. There are divergent ideas about the importance of the cytoplasmic tail and its acylation for virus maturation. Reverse genetic analyses of the HA (H7 subtype) suggested that the integrity of the cytoplasmic tail and its acylation is advantageous. It was shown that acylation-mediated membrane anchoring of HA is essential for fusion pore formation and virus infectivity (Wagner et al. 2005).

Upon transport of the HA through the Golgi apparatus, the *N*-linked oligosaccharides are enzymatically processed to more complex forms by a number of different transferases. Many studies on the relevance of glycosylation for HA function indicate that glycosylation plays an important role in the virulence of influenza A viruses. Glycosylation patterns of the HA are host cell-specific and affect folding, transport, proteolytic cleavage, receptor binding, and fusion activity of the HA (Gallagher et al. 1988; Gambaryan et al. 1998; Kawaoka et al. 1984; Mir-Shekari et al. 1997; Ohuchi et al. 1997a, b; Schulze 1997) and thereby the

infectivity of the virus. In addition, carbohydrate side-chains attached to the HA have been found to affect antigenic properties and modulate HA recognition by CD4 + T cells (Drummer et al. 1993; Munk et al. 1992).

The final maturation event of the HA, which renders the virus fully infectious and determines its ability to spread in the tissue of the infected host, is the proteolytic cleavage of the HA_0 into its subunits HA_1 and HA_2. This cleavage is absolutely essential for HA-function and cell infection. This cleavage primes the HA molecule to undergo a drastic conformational change in a low pH-environment. This structural rearrangement of the HA results in exposure of the hydrophobic *N*-terminus of the HA_2 peptide that is able to induce fusion between the viral and cellular membranes. The cleavage activation of HA is mediated by cellular or extra-cellular enzymes and determines both tropism and the clinical outcome of an influenza infection (Klenk and Garten 1994; Rott et al. 1995). A number of proteases have been identified that can activate the HA molecule depending on the amino acid sequence, accessibility and structure of the cleavage site. Some subtype H5 and H7 HA proteins containing several basic amino acid residues at their cleavage sites are activated by ubiquitous intra-cellular subtilisin-like eukaryotic endoproteases such as furin and PC6 (Horimoto et al. 1994; Stieneke-Grober et al. 1992; Walker et al. 1994). Due to the ubiquitous presence of these proteases, avian viruses possessing HAs with multibasic cleavage sites, can be produced in infectious form in most host organs and are therefore highly pathogenic (highly pathogenic avian IV = HPAIV). In contrast, HAs of the other subtypes always contain monobasic cleavage sites and are activated only after virus release by extracellular proteases. This includes trypsin (Klenk et al. 1975; Lazarowitz and Choppin 1975), a chicken endoprotease that shows homology to the blood clotting factor X (Gotoh et al. 1990), inflammatory active proteases such as kallikrein, urokinase, thrombin (Scheiblauer et al. 1992), and tryptase Clara which is secreted from cells of the bronchiolar epithelia (Kido et al. 1992, 1993; Sakai et al. 1994; Tashiro et al. 1992). Very often a combined infection of IV and bacteria like *Staphylococcus aureus*, *-pneumoniae* and *Hemophilus influenzae* occurs. The Staphylococcus protease is another serine protease-like enzyme that has the capacity to cleave HA and thereby accelerates the spread of a co-infecting influenza virus (Tashiro et al. 1987, 1992). Recently, novel type II transmembrane serine proteases (MSPL, TMPRSS13, and HAT) were identified that proteolytically activate HA membrane fusion activity and induce multi-cycle replication (Okumura et al. 2010; Bottcher et al. 2006).

After adsorption and endosomal uptake, virus disassembly occurs in the acidic environment of late endosomal vesicles and involves two crucial events. First, the conformation of the HA is changed to a low-pH form, which results in exposure of a fusion active protein sequence within the HA_2 to initiate fusion with the viral envelope. Next, the low pH in the endosomes activates the viral M2 ion channel protein resulting in a flow of protons into the interior of the virion. Acidification within the viral particle facilitates dissociation of the RNPs from the M1 protein. Vacuolar (H+)-ATPases (V-ATPases) facilitate these steps by acidifying the endosomal interior. The V-ATPase activity is mediated by IV-induced extracellular

signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K) activity (Marjuki 2010). The RNPs are subsequently released into the cytoplasm. As the RNP associated viral proteins contain nuclear localization signals (NLS), they are rapidly imported into the nucleus through the nuclear pore complexes [For review: (Palese and Shaw 2007; Wright et al. 2007)].

2.2.2 Genome Replication/Transcription

The IVs pursue a nuclear replication strategy. In order to accomplish nuclear import and export of its genome, the virus utilizes the cellular transport machinery. The transport of the RNPs into the nucleus is likely to be mediated by the viral PB2 and NP. They carry nuclear localization signals and interact with the α-class of karyopherin import receptor proteins (Neumann et al. 1997; Palese and Shaw 2007; Wang et al. 1997; Weber et al. 1998). Their nuclear import also depends on the presence of the import factors karyopherin α- and β, Ran and p10 (O'Neill et al. 1995). Furthermore, it was shown that the interaction of PB2 and NP with importin α1 is a host range determinant as adaptive mutations in both proteins enhance their binding to importin α1 and increase their transport into the nucleus of mammalian cells. In avian cells these effects were not observed (Gabriel et al. 2008).

The viral genomic segments are replicated and transcribed by the viral RdRp as part of the RNPs in the nucleus of the infected cell. The vRNA is directly transcribed to mRNA and, in addition, serves as a template for a complementary copy (cRNA), which itself is the template for new vRNA [For review: (Palese and Shaw 2007; Wright et al. 2007)]. PB1 is predicted to contain the central location of the polymerase domain. The PB2 subunit was identified as the site of cap binding. Various studies have shown that PB1-F2 has several effects: (i) it can induce apoptosis in a cell type-dependent manner; (ii) it is able to promote inflammation; and (iii) it can up-regulate viral polymerase activity by its interaction with the PB1 subunit. These properties could contribute to an enhanced pathogenicity. The underlying mechanisms are not fully understood and some effects of PB1-F2 might be strain- and host-specific (Mazur et al. 2008). The cap-snatching endonuclease of the IV RdRp resides in the PA subunit. By interaction of the C-termini of PA and PB1 with the *N*-termini of PB1 and PB2, respectively, the RdRp complex is formed. (Dias et al. 2009). The viral NP which associates with the genomic and anti-genomic viral RNAs is an essential cofactor of the viral replicative complex (Huang et al. 1990). Genomic IV RNAs carry at their 5′- and 3′-ends conserved nucleotide sequences of 13 and 12 bases, respectively. As these sequences are in part complementary, the ends of viral RNAs can engage in base-pairing interactions resulting in a partially double-stranded promoter structure (Fischer et al. 1998; Flick et al. 1996; Luo and Palese 1992). The viral RdRp binds to these promoter structures of the viral RNA segments and subsequently initiates RNA synthesis (Palese and Shaw 2007). In comparison to replication more is known about viral transcription. The RdRp does not synthesize the 5′-cap structures (m7GpppNm) that are needed for efficient transport and translation of the viral

mRNAs. Instead, the 5′-cap structures from cellular polymerase II transcripts are transferred to viral mRNAs (Krug et al. 1979). It was realized quite early on that IV is inhibited under conditions where the cellular RNA polymerase II is blocked (Rott and Scholtissek 1970). The requirement for capped mRNA 5′-ends to function as primers in viral transcription most likely explains the dependency of viral replication on RNA polymerase II activity.

In the early phase of infection the viral genome is both transcribed and replicated at high rates. At later time points (>3.5 h p.i.) transcription decreases, whereas the replication of viral RNAs continues (Shapiro et al. 1987). It is not known in detail which regulatory factor(s) participate in this process. However, analysis of viral replication in vitro suggests that a fraction of the NP which is not associated with the viral RNPs may have a regulatory role in the switch from viral transcription to replication (Shapiro and Krug 1988). Furthermore, the viral M1 matrix protein that accumulates to high levels during the late phase of infection has been shown to inhibit viral transcription (Hankins et al. 1989; Perez and Donis 1998; Ye et al. 1987). M1 may therefore also be involved in the down regulation of viral mRNA synthesis late in infection.

2.2.3 Controlling the Cell

The NS segment encodes the NS1 protein, which is translated from unspliced mRNA, as well as the NEP protein, which is translated from spliced mRNA transcripts. The NS1 protein has been shown to be a major pathogenicity factor [For review: (Hale et al. 2008)]. As such, it can impair host innate and adaptive immunity in a number of ways. It can block the function of 2′-5′-oligoadenylate synthetase (OAS) (Bergmann et al. 2000) and bind to double-stranded RNA (dsRNA), thereby suppressing the activation of ds RNA-activated protein kinase (PKR), both important regulators of translation that can induce the host apoptotic response and type I interferon (IFN) production (Marjuki et al. 2007). Furthermore, it can inhibit retinoic acid-inducible gene I (RIG-I)-mediated induction of IFN by: (i) binding to RIG-1, and preventing it from binding to single-stranded RNA (ssRNA) bearing 5′-phosphates (Qian et al. 1994); or (ii) forming a NS1/RIG I-RNA complex (Falcon et al. 2004); or (iii) interacting with the ubiquitin ligase TRIM25 and inhibiting TRIM25-mediated RIG-I CARD ubiquitination (Gack et al. 2009). Another way by which NS1 impairs the production of IFN is to prevent the activation of transcription factors such as ATF-2/c-Jun, NF-κB, and IRF-3/5/7, all of which stimulate IFN production (Gambotto et al. 2008; Koopmans et al. 2004). By forming an inhibitory complex with NXF1/TAP, p15/NXT, Rae1/mrnp41, and E1B-AP5, which are important factors in the mRNA export machinery, NS1 decreases cellular mRNA transport in order to render cells highly permissive to IV replication (Robb et al. 2009). NS1 can also inhibit the 3′-end processing of cellular pre-mRNAs (including IFN pre-mRNA) through interaction with the cellular proteins CPSF30 (Treanor et al. 1989) and PABII (Bergmann et al. 2000).

Additionally, many studies have highlighted the importance of the interaction between the RNP complex and NS1 for viral replication (Donelan et al. 2003; Krug et al. 2003; Maines et al. 2005; Mibayashi et al. 2007). The NS1 protein was shown to interact with the RNP complex in vivo (Maines et al. 2005), and truncated NS1 affected production of vRNA, but not of cRNA and mRNA in infected MDCK cells, implicating NS1 in the regulation of replication (Donelan et al. 2003). The NS1 protein was also found to regulate the time course of viral RNA synthesis during infection as a mutant virus with two amino acid changes at positions 123 and 124 deregulated the normal time course of viral RNA synthesis (Mibayashi et al. 2007). Interestingly, characterization of H7-type HPAIV with reassorted NS segments from H5- and H7-type avian strains, generated by reverse genetics, demonstrated altered growth kinetics of the reassortant viruses that differed from the wild type. Surprisingly, the effects differed between cells of mammalian or avian origin; and molecular analysis revealed that the reassorted NS segments were not only responsible for alterations in the anti-viral host response, but furthermore affected viral genome replication and transcription as well as nuclear RNP export. IFN-beta expression and the induction of apoptosis were found to be inversely correlated with the magnitude of viral growth, while the NS allele, virus subtype and levels of NS1 protein expression showed no correlation. Thus, these results demonstrate that the origin of the NS segment can have a dramatic effect on the replication efficiency and host range of HPAIV. Overall, these data suggest that the propagation of NS reassortant IV is affected at multiple steps of the viral lifecycle as a result of the different activities of the NS1 protein on multiple viral and host functions (Wang et al. 2010). The NEP protein which interacts with the viral M1 protein and mediates the export of vRNPs from the nucleus to the cytoplasm (Nemeroff et al. 1998), has also been shown to play a role in the regulation of viral replication and transcription. However a direct interaction between NEP and the viral polymerase complex has not been demonstrated. Nevertheless, it is evident that both the NS1 and NEP proteins play important roles in viral pathogenicity and replication.

2.2.4 Assembly and Release

In the late phase of the replication cycle newly synthesized viral RNPs are exported to the cytoplasm, and two viral proteins have been suggested to play important roles in this transport event. It has been observed that the appearance of the viral M1 protein in the nucleus is required for the subsequent export of viral genomes, but the molecular basis of this dependency remains elusive (Subbarao et al. 1998). The M1 protein is known to associate with viral nucleocapsids and the NEP protein. It has been shown that the NEP protein contains a leucine-rich nuclear export signal by which it interacts with nucleoporins (Weber et al. 1998). In light of this finding, it was suggested that the M1-NEP complex mediates the export of associated viral nucleocapsids in the late phase of the replication. Previously, it was demonstrated that IV activates the Raf/MEK/ERK-signal transduction cascade, which is an

essential pre-requisite for efficient nuclear RNP export (Ludwig et al. 2004; Pleschka et al. 2001). Surprisingly, other IV-induced events strongly contribute to the nuclear RNP export, namely the activation of NF-kB, as well as the apoptotic activity of caspase3 (Wurzer et al. 2003, 2004). As these cellular factors and mechanisms are essential for IV replication, their inhibition strongly affects IV replication. Because they are encoded in the host genome, the virus can hardly become resistant by mutation, making them ideal targets for therapeutic intervention (Ludwig et al. 2003). In the cytoplasm, the M1 protein inhibits re-import of the nucleocapsids into the nucleus, possibly by masking the karyophilic signals of the NP (Bui et al. 1996). Another mechanism of cytoplasmic retention of the RNPs was proposed to be due to the ability of NP to associate with the actin cytoskeleton (Avalos et al. 1997; Digard et al. 1999).

The mature HA and NA glycoproteins and the nonglycosylated M2 are finally integrated into the plasma membrane as trimers (HA) or tetramers (NA, M2), respectively. M1 assembles in patches at the cell membrane. It is thought to associate with the glycoproteins (HA and NA) and to recruit the RNPs to the plasma membrane in the late phase of the replication cycle. Finally, the viral RNPs become enveloped by a cellular bi-lipid layer carrying the HA, NA, and M2 proteins resulting in budding of new virus particles from the apical cell surface. Interestingly, HA membrane accumulation actually triggers the essential ERK signaling. This represents an auto-regulative feedback loop that assures nuclear RNP export at a time point when all other viral components are ready for budding (Marjuki 2006). Furthermore, higher polymerase activity of a human IV enhances activation of the HA-induced Raf/MEK/ERK-signal cascade resulting in more efficient nuclear RNP-export as well as virus production (Marjuki et al. 2007).

By the receptor destroying neuraminidase activity of the NA, the progeny virions are able to detach from the cell surface, to which they would otherwise reattach by the HA activity. As such the NA, which is a type II glycoprotein that follows the exocytotic transport pathway and presumably encounters many of the same enzymes that HA does, plays an essential role in release and spread of progeny virions. Moreover, NA was also shown to be important for the initiation of IV infection in human airway epithelium (Matrosovich et al. 2004). A functional balance between HA and NA in IV infections seems to be highly important. Both proteins recognize sialic acid. HA binds to sialic acid-containing receptors on target cells to initiate virus infection, whereas NA cleaves sialic acids from cellular receptors to facilitate progeny virus release. Studies have revealed that an optimal interplay between these receptor-binding and receptor-destroying activities of HA and NA is required for efficient virus replication. An existing balance between the antagonistic HA and NA functions can be disturbed by reassortment, virus transmission to a new host, or therapeutic inhibition of NA. The resulting decrease in the viral replicative fitness can be overcome by restoration of the functional balance via compensatory mutations in HA, NA or both proteins (Wagner et al. 2002).

2.3 Antigenic Drift and Antigenic Shift

The polymerase complex of IV does not possess a proof reading activity, thus numerous mutations accumulate in the viral genome during ongoing replication (Palese and Shaw 2007) leading to changes in all proteins. This includes conformational alteration of HA- and NA-epitopes against which neutralizing antibodies are generated. Influenza A viruses are categorized by antigenic differences of the HA and NA-proteins. The high mutation rate combined with the high replication rate results in a multitude of new variants produced in each replication cycle, thus allowing the virus to rapidly adapt to changes in the environment. This results in an escape of the existing immunity and in resistance to drugs acting directly against viral functions. Gradual changes of the antigenic properties that make existing vaccines less or noneffective are described as *antigenic drift* and demand for new compositions of the yearly vaccines.

Due to the nature of their segmented genome, IV can independently recombine segments upon the infection of a cell with two different viruses. This is described as *genetic reassortment*. Through its receptor binding and fusion activity as well as its antigenic properties, the HA is a major determinant of tissue tropism, viral spread, and pathogenicity in IV-infected organisms. Today 17 HA-subtypes (H1–H17) and 9 NA-subtypes (N1–N9) are known, which can mix and lead to new antigenic properties (Palese and Shaw 2007; Webster et al. 1992; Wright et al. 2007). Not all combination will ultimately be advantageous, but can lead to the generation of a virus that combines the ability to replicate in humans with novel antigenic properties (*antigenic shift*). This has happened at least three times in the last century, resulting in the pandemics of 1918 ("Spanish Flu"), 1957 ("Asian Flu") and 1968 ("Hong Kong Flu") that together caused up to 40 million deaths. The 2009 introduction of the new pandemic H1N1-type swine origin IV (S-OIV) into the human population, which comprises a reassortant IV harboring segments of human and avian IV, as well as of swine IV belonging to the North American and Eurasian lineage demonstrates that the question is not "if" but "when" will such new pandemics occur (Horimoto and Kawaoka 2001; Webby and Webster 2003; Webster 1997).

Besides pandemic variants that can occur when human and avian IV reassort in porcine hosts (regarded as "mixing vessels") (Webster et al. 1995; Webster 1997a, b), HPAIV strains have directly infected humans, as happened in Hong Kong in 1997 (Claas et al. 1998; de Jong et al. 1997; Subbarao et al. 1998), and thereafter during vast outbreaks of avian influenza (Fouchier et al. 2004; Koopmans et al. 2004). These viruses show an extremely high virulence in humans with case fatality rates up to 60 % (World Health Organization 2005).

2.4 "Flu"- the Disease

The virus that usually causes a respiratory disease in humans [For references: (Wilschut 2005)] is transmitted by aerosol droplets and contaminated hands and can already be shed before the onset of symptoms (Cox et al. 2004). Therefore, high population density and dry air leading to reduced protection of respiratory epithelium by the mucus are conditions that promote transmission of the virus.

The infection with IV in humans is normally limited to the respiratory tract. Here, proteases released in the epithelium are present that activate the HA to allow further infections (Sect. 2.2.1) [For review (Ludwig et al. 1999)]. Innate immunity as well as the adaptive immune system will normally restrict virus propagation. Therefore, population groups which have a less protective immune system, such as young children up to 2 years and older persons over 65 as well as immunocompromised or chronically diseased persons, are especially at risk. The replication of the virus leads to the lysis of the epithelial cells and enhanced mucus production, causing runny nose and cough. Also, inflammation and edema at the replication site due to cytokines released contribute to the disease. This can lead to fever and related symptoms. Bacterial superinfections of the harmed tissue can further complicate the situation. Normally, onset of systemic (fever, myaglia, headaches, and severe malaise) and respiratory (coughing, sore throat, and rhinitis) symptoms occurs after about 2 days incubation period and can last for about 7 to 10 days. Coughing and overall weakness can persist for up to 2 weeks. If the virus spreads from the bronchiolar tract to the alveoli, viral pneumonia and interstitial pneumonitis with mononuclear and hemorrhage infiltration and finally lysis of the intealveolar space are all possible (Wilschut 2005).

This scenario is a likely picture in case of infection with a pandemic IV strain, where the individual has not had a prior exposure to the virus and the innate immunity reaction can lead to a strong immune reaction. High virus replication will induce secretion of large quantities of cytokines by the infected epithelia and will stimulate inflammatory processes. Together with the destruction of the epithelia, this results in an influx of fluids into the alveoli leading to hypoxia and acute respiratory distress syndrome that may cause the death within a short period of time (1–2 days after onset). This scenario might also be caused by additional viral factors enhancing pathogenicity. Such factors that are not yet well defined but probably contributed to the devastating outcome of the "Spanish Flu" (Wilschut 2005).

Accurate and rapid diagnosis of the disease is essential for effective treatment, especially with anti-viral substances, as virus replication and therefore illness progresses rapidly. Samples can be tested serologically, by cell culture or RT-PCR for strain typing and should be done within days after onset of symptoms (Wilschut 2005).

Since pandemic virus strains usually possess unique antigenic characteristics, current vaccines will be ineffective once such a virus emerges. Regarding the vast possibilities for such a novel strain to "travel" around the world (Hufnagel et al. 2004), it becomes evident that effective countermeasures are required for the fight

against these foes. In recent outbreaks of avian viruses that infected humans (Chen et al. 2004; Hatta and Kawaoka 2002; Li et al. 2004) a mortality rate of about 60 % was observed (World Health Organization 2005). Fortunately, until now these particular viruses have not acquired the ability to spread in the human population. However, any novel virus strain emerging in the future may have such a capability (Webby and Webster 2003).

As every virus depends on its host cell, cellular functions essential for viral replication may also be suitable targets for anti-viral therapy. In this respect, intracellular signaling cascades activated by the virus, in particular MAPK pathways, have recently come into focus (see Sect. 2.2.4) (Ludwig et al. 1999, 2003).

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History of Swine Influenza

Stacey Schultz-Cherry, Christopher W. Olsen and Bernard C. Easterday

Abstract Swine influenza is a continual problem for the swine industry and can pose a public health threat as evidenced by the 2009 H1N1 influenza virus pandemic. Given its importance, it is not surprising to find papers describing the disease from the early 20th century. In this chapter, we discuss the history of swine influenza, the important role swine influenza virus has played in our understanding of influenza virus pathogenesis and virology, and its impact on public health worldwide.

Contents

Swine influenza (SI) has been a commonly recognized disease of swine for more than 90 years. Despite considerable interest and research efforts over the past 50 years, SI continues to be an important economic issue in swine production in diverse parts of the world today. Given the 2009 H1N1 pandemic, which was unfortunately known as "swine influenza" or "swine flu", it seems appropriate to review the origin of this important disease of swine, its importance in the swine production industry, and its relationship to influenzas of humans and other animals.

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Current Topics in Microbiology and Immunology (2011) 370: 21–27
DOI: 10.1007/82_2011_197

Published Online: 21 January 2012

Most reviews on SI will begin with some historical descriptions of when and where the disease was first observed. The earliest papers are from the early twentieth century. Koen (1919) stated that "Than the differential diagnosis of swine diseases there is no more important subject confronting the veterinary profession today" in "A Practical Method for the Field Diagnosis of Swine Diseases". Koen was a Bureau of Animal Industry inspector (USDA), in charge of hog cholera control work in Iowa. In that capacity he had abundant opportunity to investigate a variety of diseases of swine. He emphasized that his approach was "practical" diagnosis that was based on three things: history, clinical signs, and post-mortem lesions. He described the differential diagnosis of five conditions including "flu". Remarkably, his descriptions of "flu" in swine would match the appearance of any current SI natural outbreak. His comments in his closing arguments bring an intriguing perspective on the 1918 pandemic. He wrote that "Last fall and winter [1918] we were confronted with a new condition, if not a new disease. I believe I have as much to support this diagnosis in pigs as the physicians have to support a similar diagnosis in man. The similarity of the epidemic among people and the epizootic among pigs was so close, the reports so frequent, that an outbreak in the family would be followed immediately by an outbreak among the hogs, and vice versa, as to present a most striking coincidence if not suggesting a close relation between the two conditions. It looked like 'flu', it presented the identical symptoms of 'flu', it terminated like 'flu' and until proved it was not 'flu', I shall stand by that diagnosis". However, a 1921 report by Dr. Charles Murray, Professor of Veterinary Medicine, Iowa State College, presented an opposing view in an article entitled "WHAT IS HOG 'FLU'" (Murray 1921). He wrote that the disease in swine "…was unfortunately given the name of 'hog flu', a name which caused much apprehension among the agricultural people who were led to believe through the similarity of names that the diseases were the same cause and that the one affecting swine was transmissible to man. Such was not the case, and no such transmission ever occurred."He also stated that "So called 'swine flu', a name which, while it became quite popular through its association with the human disease, is nevertheless a misnomer…".

The issue of calling the disease "swine flu" led to continued debate at the annual meeting of the American Veterinary Medical Association in 1922 where a paper entitled "Remarks on 'Hog Flu'" was presented (Dorset et al. 1922). This group acknowledged that the name 'flu' given by Koen seemed to be appropriate, but suggested that the name 'hog flu' should be used to avoid confusion with human influenza. We were confronted with this issue more than 90 years later during the 2009 H1N1 pandemic where misunderstandings associated with the name "swine flu" led to considerable losses to the swine industry. Despite consistent and clear evidence to the contrary, people became fearful of catching pandemic influenza by eating pork (Joint FAO/WHO/OIE 2009).

Regardless of the name, SI continued to be a problem in swine herds throughout the twentieth century. In 1927, McBryde reported on the nature of the disease including the sudden onset with the entire herd having the disease within a couple of days. Clinical signs noted (similar to today) included fever, loss of appetite,

lethargy, "thumpy" respiration, cough, loss of condition, and low mortality followed by quick recovery (McBryde 1927). Koen (1919) and other authors throughout the 1920s consistently reported that outbreaks of SI were associated with high morbidity and low mortality, which is also consistent with the disease as observed today. A major advance during that time was the ability to differentially diagnose hog cholera and swine influenza. As related to Dr. Easterday by the late S.H. McNutt, former mentor and colleague, a crude field method of differentiation was based simply on mortality patterns—"We finally determined how to differentiate between hog cholera and swine influenza—if most of the affected animals died they had cholera and if most of them lived they had flu."

Historically, influenza in swine was a disease of late autumn and early winter. However, with contemporary swine production methods including extensive climate control and animal confinement, the disease now can appear throughout the year rather than only seasonally. In the early years of the disease the farmers were very concerned and called their veterinarians on a regular basis for help. However, after learning that there was no specific treatment and that the animals would typically recover with little or no death loss, this has become a disease that is managed in many cases without direct veterinary medical input.

Even in the 1920s it was not uncommon to meet farmers and veterinarians who believed that they had contracted the disease from affected swine. In 1928, McBryde et al. reported on the transmission of SI by infectious materials from an affected animal to a normal animal and by placing normal animals with affected animals (McBryde et al. 1928). They concluded that the infectious agent was in nasal, tracheal, and bronchial fluids and that the infectious agent was not in the blood. They speculated that because of the sudden onset and that a large number of animals are simultaneously affected, some organism already present in the respiratory tract was the causative agent and was activated when the resistance of the animals was lowered by the harsh environment. They believed that there was little if any immunity, because "…it seems to be quite well established that the same herd may suffer from two or even three attacks of flu in one season." In 1931, Shope proved that swine influenza was caused by a virus and that he could reproduce SI under strict experimental conditions by inoculating both filtered and unfiltered material from affected pigs into the respiratory tract of normal pigs (Shope 1964). Subsequently, Shope would describe SI as a "…disease of complex etiology, being caused by infection with the bacterium *H. influenzae suis* and the swine influenza virus acting in concert." (Although we now understand that the virus alone is sufficient to cause the disease). Subsequently, in 1934, Andrewes et al. confirmed that human influenza was also associated with a virus (Andrewes et al. 1934).

Shope's work in the 1930s and 1940s was instrumental in our understanding of influenza. He demonstrated that SI could infect mice (Shope 1935) and ferrets (Shope 1934) and that humans had neutralizing antibodies for SI (Shope 1936), suggesting that the human influenza virus was antigenically similar to the swine influenza virus. Particularly intriguing was his hypothesis on how SI was maintained in nature as a seasonal disease. While the *H. influenzae suis* bacterium could

be found throughout the year, with the methods available at the time the virus was not demonstrated during the 9-month interepizootic period. He hypothesized that the virus was harbored and transmitted in swine lungworms (*Metastrongylus*) and performed experiments which he believed demonstrated that the virus remained in an occult or masked form in the lungworms and was provoked to infectivity by an adverse meterological condition. However, the virus could not be detected by direct means in the lungworm larvae, in the earthworm intermediate host, or in the adult lungworm in the definitive host (Shope 1941a, b, 1943a, b, 1955). Nonetheless, that hypothesis served to stimulate many to further investigate SI. It is now recognized that the virus circulates throughout the year and that there is no conclusive evidence for a complex invertebrate host system to maintain the disease.

The unresolved question of the relationship of swine and human influenza and their respective viruses fueled speculation on the role of animals in human influenza. When the human pandemic of 1957 first began to spread in Asian countries, the World Health Organization (WHO) decided to perform an animal serum survey to better understand the role of animals in the epidemiology of influenza (Kaplan and Payne 1959). This decision was based on reports from China of epizootics of influenza-like disease in swine in areas severely affected by the human disease. Veterinary medical service agencies in 33 countries throughout the world participated in the survey by collecting serum from swine and horses. As requested by the WHO, the testing laboratories performed complement–fixation and hemagglutination-inhibition tests with provided reagents. These studies demonstrated that the Asian ("A2" at the time and in the old influenza nomenclature) strain caused naturally occurring, but often inapparent, infection in horses and swine. And following recognition of infection in pigs with the A-swine strain, long known in the USA, in Germany, and Czechoslovakia (Kaplan and Payne 1959), Kaplan and Payne (1959) stated that "For the clarification of the natural history of influenza, so urgently needed, this problem of animal influenza can no longer be neglected. Investigations along the lines indicated above will certainly add much to our knowledge of influenza epidemiology, and the World Health Organization hopes to stimulate and coordinate such studies in the future".

The WHO Expert Committee on Respiratory Virus Diseases met in 1958 and devoted most of the five-day meeting to influenza (Expert Committee on Respiratory Virus Diseases 1959). They considered the relationship between Asian influenza virus and viruses infecting animals, and the role, in general, of animals in the epidemiology of human influenza. During the course of that meeting, a combined session with the Joint WHO/FAO Expert Committee on Zoonoses was held. As a result of the considerable discussions, both independently and together, both agencies concluded that further investigations on the relationship between human and animal influenza viruses were needed. In 1960, Steele proposed the possibility of an animal reservoir for influenza A viruses may exist in nature (Steele 1961) and greater emphasis thereafter was placed on surveillance in animals.

Dr. Martin M. Kaplan served as the first Chief of Veterinary Public Health for the WHO from 1949 to 1969 and Director of the Office of Science and Technology

(eventually to be called the Office of Research Promotion and Development) from 1969 until his retirement in 1976. He provided strong international leadership in influenza surveillance in animals and organized a worldwide survey in 1957. Kaplan convened the first WHO Informal Meeting on the Coordinated Study of Animal Influenza in Prague in January 1963. Such meetings were expanded and continued into the early 1980s. Because of the continued prominence of questions about the role of swine and swine influenza virus in human influenza, the participants in that first meeting agreed that the major efforts should be directed toward epidemiological studies in swine and to standardizing test reagents and technical procedures. The technical procedures focused on minimizing the possibility of laboratory contaminations in virus isolation and providing standard reagents and procedures for serological studies to reduce the problems of nonspecific inhibitors in animal sera. Collaborators were expected to provide data on a regular basis to Dr. Kaplan and he, in turn, summarized the material and provided it to all of the collaborators.

The next meeting occurred in 1964 and the major topics of discussion at that meeting were swine influenza and equine influenza with other areas being avian influenza and the surveillance in mammals other than swine and horses. Antigenic analysis of influenza viruses was becoming a larger part of the collaboration, but the group agreed that for a variety of reasons, including the pitfalls of serological testing, there needed to be greater emphasis on the isolation of viruses.

Although significant research with swine and swine influenza continued, an increasing number of isolations of influenza viruses from a variety of avian species led to expanded collaborations on the epidemiology of influenza in birds. WHO was able to provide support for some part of the research activities at most of the collaborating laboratories (Kaplan 1969); however, it should be recognized that a major basis for the success of these collaborations was the collegiality and trust that developed among the participants. This included generous sharing of data as it was developed and opportunities to visit the various participating laboratories and institutions. The collaborative efforts resulted in many joint publications involving participants from two or more laboratories.

By 1970 considerable evidence had accumulated, based on serological studies, that people whose occupations brought them in contact with swine became infected with the swine influenza virus. In 1974, the swine influenza virus was isolated from the lung of a boy who had died with Hodgkin's disease (Easterday 1986). He had been in contact with swine five days before he died and the swine had antibody to the virus. An acute respiratory disease in an 8-year-old boy in Wisconsin in 1975 was attributed to infection with swine influenza virus based on serological studies of serum from the boy and the swine with which he had been in contact (Easterday 1986). Then came the Fort Dix, New Jersey swine influenza virus "episode" beginning in January 1976 in which recruits fell ill with respiratory illness ultimately shown to be due to infection with a swine influenza virus (Gaydos et al. 2006). All speculation about the transmission of the virus from swine to human beings came to an end when the virus was isolated from swine and their caretaker on a Wisconsin farm in the autumn of 1976 (Easterday 1986), and a

recent comprehensive review article by Myers et al. documents multiple cases of human infection with SI viruses since that time (Myers et al. 2007).

Much remains to be learned about SI as a disease of swine. It continues to be a problem in swine production and the high morbidity rate with acute illness in the swine is estimated to result in a delay of as much as two weeks in the affected animals reaching market weight. That delay results in increased production costs. Thus, efforts have been aimed toward prevention of infection. Swine influenza vaccines have been used commercially in swine herds in the US since the early 1990s. During the past several years, with the emergence of multiple subtypes and genotypes not previously circulating in swine in the US, manufacturers have updated the composition of the vaccines to include the new strains. Due to documented cases of reverse zoonotic transmission of the 2009 pH1N1 virus to pigs, this included development of a USDA conditionally licensed pandemic H1N1 strain vaccine for pigs. The choice to vaccinate in the US remains a decision between producers and their herd veterinarians. In addition to the commercially available vaccines in the US, it has been estimated that more than 50% of the vaccines that are used are autogenous products that are custom-created for individual swine production units. Swine are also vaccinated against SI in many other parts of the world, though the specific virus strains in vaccines and role of vaccination vary with the swine production management systems.

Prior to the late 1990s, the 1918-derived H1N1 virus was the predominate agent of SI circulating among swine in North America. However, since the late 1990s, we have seen the emergence of novel strains of influenza viruses in swine in North America, including most prominently, triple reassortant H3N2, H1N2, and H1N1 viruses (reviewed in Olsen 2002; Vincent et al. 2008), as well as wholly avian H4N6, H3N3, and H1N1 swine isolates (Karasin et al. 2000, 2004). These events, as well as the recognition that the 2009 pandemic H1N1 virus had its genetic origins in viruses of swine influenza origins, should serve as a wake-up call for the world's animal health and public health communities. One must remain vigilant to the constant emergence of new influenza viruses in animals and surveillance of influenza in animals and humans would be well served by an integrated system and "one health" approach.

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Genetics, Evolution, and the Zoonotic Capacity of European Swine Influenza Viruses

Roland Zell, Christoph Scholtissek and Stephan Ludwig

Abstract The European swine influenza virus lineage differs genetically from the classical swine influenza viruses and the triple reassortants found in North America and Asia. The avian-like swine H1N1 viruses emerged in 1979 after an avian-to-swine transmission and spread to all major European pig-producing countries. Reassortment of these viruses with seasonal H3N2 viruses led to human-like swine H3N2 viruses which appeared in 1984. Finally, human-like swine H1N2 viruses emerged in 1994. These are triple reassortants comprising genes of avian-like H1N1, seasonal H1N1, and seasonal H3N2 viruses. All three subtypes established persistent infection chains and became prevalent in the European pig population. They successively replaced the circulating classical swine H1N1 viruses of that time and gave rise to a number of reassortant viruses including the pandemic (H1N1) 2009 virus. All three European lineages have the capacity to infect humans but zoonotic infections are benign.

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Current Topics in Microbiology and Immunology (2013) 370: 29–55
DOI: 10.1007/82_2012_267

Published Online: 26 September 2012

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1 Introduction

Swine influenza was first recognized as a disease of pigs during the great pandemic in autumn 1918. At that time, John S. Koen, who worked as a hog cholera inspector for the U.S. Bureau of Animal Industry in Fort Dodge, Iowa, observed a striking similarity between the clinical presentation of diseased humans and pigs: "*Last fall and winter we were confronted with a new condition, if not a new disease. I believe I have as much to support this diagnosis in pigs as the physicians have to support a similar diagnosis in man. The similarity of the epidemic among people and the epidemic in pigs was so close, the reports so frequent, that an outbreak in the family would be followed immediately by an outbreak among the hogs and* vice versa*, as to present a most striking coincidence if not suggesting a close relation between the two conditions. It looked like "flu", it presented the identical symptoms of "flu", it terminated like "flu", and until proved it was not "flu", I shall stand by that diagnosis.*" (Koen 1919). The etiologic agent of "flu", influenza A virus, was first isolated by Richard E. Shope (Shope 1931a).

Influenza viruses are members of the family *Orthomyxoviridae* which comprises five genera: *Influenza virus A*, *B*, and *C*, *Thogotovirus*, and *Isavirus* (Kawaoka et al. 2005). Each influenza virus genus includes one species (also designated as *influenza A virus*, *influenza B virus*, and *influenza C virus*; abbreviated FLUAV, FLUBV, FLUCV).

Influenza A viruses are enveloped negative-stranded RNA viruses. The RNA genome is segmented (Duesberg 1968) and associated with the viral nucleoprotein

(NP) and the viral polymerase complex. The eight RNA segments vary in their sizes (ranging from 890 to 2,341 nucleotides) and encode 11 proteins. Expression of viral genes occurs after transcription of genomic RNA with the help of the viral RNA-dependent RNA polymerase.

New isolates of influenza A virus predominantly have a filamentous structure with a diameter of approximately 80–120 nm and a length ranging from 2 to 200 μm (Chu et al. 1949). After adaptation to cell culture, the virion tends to have a spherical or pleomorph appearance. The viral envelope is composed of the cell membrane lipids but the majority of surface proteins are provided by the viral hemagglutinin (HA) and neuraminidase (NA). A third viral membrane protein is the M2 proton channel. The inner surface of the envelope is coated with the matrix protein (M1). The virion contains eight nucleocapsids; these are complexes of RNA and viral protein. Electron micrographs show helical rod-like structures with a terminal loop. The width ranges from 10 to 15 nm and the length from 30 to 120 nm. They were interpreted to represent backfolded and twisted ribonucleoproteins (Compans et al. 1972). The nucleocapsids are associated with the envelope by matrix proteins (Noda et al. 2006).

2 Influenza Virus Ecology

Influenza A viruses have a broad host range (Webster et al. 1992). The main reservoir hosts are aquatic birds of the orders *Anseriformes* (geese and ducks) and *Charadriiformes* (waders and gulls), but numerous other bird species may also be infected (Munster et al. 2007). Reassortment of 16 HA and nine NA types allows the formation of maximal 144 HA/NA combinations of which more than 110 types have been already isolated from birds. Whether all theoretical combinations exist in nature is unknown. In mammals, stable infection chains are observed only for certain subtypes (Table 1). Important mammalian host species include: humans, pigs, and horses. Dogs, domestic cats, and felid carnivores (tiger, leopard) as well as several mustelid carnivores (ferret, stone marten, mink), marine mammals (whales, seals), the camel, the muskrat, civet, racoon dog, pika, and giant anteater were described as accidental hosts without establishment of stable infection chains.

Influenza virus ecology is strongly influenced by virus adaptation to its host. One major determinant of the host range is the receptor molecule on the surface of the host cell. Influenza A virus binds to sialic acid (N-acetylneuraminic acid) which is linked by an α-glycosidic bond to the terminal galactose residues of carbohydrate chains of glycoproteins and glycolipids (Rogers and Paulsen 1983). Both species and tissue-specific expression of receptor molecules determine host range and tropism of influenza A viruses (Ito et al. 1998; Ito and Kawaoka 2000). Whereas avian influenza viruses bind to α-2,3-linked sialic acid, seasonal influenza virus strains of humans recognize α-2,6-linked sialic acid. Airway epithelia of the upper respiratory tract of pigs express both receptors. Thus, pigs are susceptible to

Table 1 Mammalian host species of influenza A virus

Host	Stable infection chain	Incidental zoonotic infection or reassorted isolate	Extinct
Human	H1N1, H3N2, pandemic (H1N1) 2009 virus	H1N2, H5N1, H7N1, H7N2, H7N3, H7N7, H9N2	H2N2
Pig	H1N1, H1N2, H3N2	H1N7, H2N3, H3N1, H3N3, H4N6, H5N1, H5N2, H9N2, pandemic (H1N1) 2009 virus	
Horse	H3N8	H1N8, H3N3	H7N7
Dog		H3N8, H5N1	
Racoon dog (*Nyctereutes procyonoides*)		H5N1	
Mink		H3N2, H10N4	
Stone marten		H5N1	
Ferret		H1N1	
Seal (*Phoca vitulina*)		H3N3, H4N5, H7N7	
Whale		H1N3, H13N2, H13N9	
Camel		H1N1	
Giant anteater		H1N1	
Tiger, leopard		H5N1	
Domestic cat		H5N1, pandemic (H1N1) 2009 virus	
Civet		H5N1	
Pika (*Ochotona spec.*)		H5N1	
Muskrat (*Ondatra zibethicus*)		H4N6	

influenza viruses which are adapted either to birds or to humans and can serve as intermediate hosts after trans-species infections (Ito et al. 1998). Due to their receptor configuration, pigs were considered as mixing vessels for the reassortment of human and avian influenza viruses (Scholtissek et al. 1985). Other host determining factors are the nucleoprotein (Scholtissek 1990) and polymerase subunit PB2. Amino acid position 627 of PB2 was shown to be critical for virus replication (Subbarao et al. 1993). The tissue-specific expression of host proteases, however, contributes to virulence or pathogenicity but not to the host range (Webster et al. 1992; Steinhauer 1999). Recent genome-wide RNAi screening studies revealed the involvement of hundreds of host factors that are required for efficient influenza virus replication (König et al. 2010; Karlas et al. 2010). It remains to be elucidated which of these factors establishes the host range.

3 Genetic Drift and Reassortment: Two Mechanisms for the Generation of Genetic Variability of Influenza viruses

A typical property of influenza A viruses is their great variability which is mainly caused by two mechanisms. Genetic drift is the continuous accumulation of nucleotide substitutions over time. The substitution rate of influenza viruses ranges from 10^{-5} to 10^{-6} substitutions/site/replication cycle depending on the experimental setup (e.g., Stech et al. 1999; Nobusawa and Sato 2006; Parvin et al. 1986). According to the genome size of appr. 13,600 nucleotides, between 1 and 10 % of the progeny virus has substitutions. Older estimations determined even higher substitution rates (e.g., Yewdell et al. 1979; Holland et al. 1982). The most base substitutions are neutral, this means they do not cause changes of the amino acid sequence or—if so—a substitution does not seem to influence the fitness of the progeny virus. The reason is that the majority of amino acid residues of influenza virus proteins are negatively selected (purifying selection). Substitutions of such amino acids would decrease the viral fitness and are only endured as long as certain selection pressures act on the virus. A host change could induce such a selection pressure. Only very few sites are positively selected. A positive selection increases the heterogeneity of the gene pool, it is also designated as 'diversifying selection'. One example of positive selection is the gradual changing of the antigenic sites of the hemagglutinin known as 'antigenic drift'. Substitutions that result in immune escape variants have an increased probability to infect hosts with preimmunity. Eighteen codons of the hemagglutinating HA1 domain were identified to be positively selected (Bush et al. 1999a, b). One driver of antigenic drift is the receptor binding avidity of the viral hemagglutinin (Hensley et al. 2009). Though antigen drift of the hemagglutinin may be striking and can be investigated by serological means, substitutions of all influenza virus genes occur with the same frequency. Accordingly, the whole genome is subjected to a genetic drift rather than antigenic drift of the HA gene only. Due to the preponderance of either positive or negative selection acting on each gene, the relation of synonymous and non-synonymous substitutions of the eight gene segments differs.

The second important mechanism of influenza virus variability is reassortment or the exchange of one or more gene segments. Reassortments are of biological importance as they lead to novel combinations of genome segments which have been evolved by negative or positive selection. This mechanism greatly enhances evolutionary rates and accounts for rapid viral adaptation to changing environmental conditions. Reassortments occur naturally or can be induced experimentally (Kilbourne 1968). They are accomplished by a segmented virus genome and by double or multiple infections of a host with virus strains of different subtypes or genetic lineages. Reassortment events leading to exchanges of HA and NA genes are of special importance as they can lead to an antigen shift. Shift variants exhibit major differences of antigenic epitopes and less cross-reactivity with pre-existing antibodies of a host. Circulation of two or more subtypes within a population at the same time can lead to reassortments which are not associated with an antigen shift.

In addition, reassortments may occur after incidental zoonotic infections. Such events introduce genes into a virus population that are adapted to other species. Beside the HA and NA genes, other gene segments can also reassort but were in the dark for long time due to a lack of sequence data. Such reassortants are serologically inconspicuous.

4 The Concept of Genetic Influenza A Virus Lineages

Genetic drift did not only contribute to the evolution of the known HA and NA subtypes, but led to the formation of distinct genetic lineages of all genome segments (Webster et al. 1992). The genetic configuration of an influenza virus is defined by its genotype (Lu et al. 2007), which describes a virus with greater accuracy than subtyping by the HA and NA types only. Precise genotyping requires complete genome sequences but greatly enhanced our understanding of influenza virus ecology and evolution. Whereas sequence comparisons of different HA types yield nucleotide identities of roughly 56 % on average, sequences of a given lineage have nucleotide identities greater than 90 %. Two factors determine the evolution of genetic lineages: host species barriers (Kuiken et al. 2006) and geographic isolation. Starting with the pandemic of 1918, two stable infection chains of H1N1 were established in humans and pigs which lead to new, distinct genetic lineages: the seasonal H1N1 of humans and the classical swine H1N1 lineage (Fig. 1). Such lineages can be demonstrated for all eight genome segments. There are distinct lineages for birds, humans, pigs, and horses. It appears that some lineages became extinct but the significance of this observation is yet unclear due to a lack of sufficient sequence data. Viruses of a lineage are adapted to their host species. Trans-species infections occasionally occur, but virus replication is less efficient and infection chains may disrupt after a few generations. Stable infection chains will establish rarely. Besides the pandemic virus of 1918, the 'avian-like' swine influenza viruses in Europe are another example for a successful establishment of a stable infection chain. However, after some 30 years of circulation the latter viruses have not yet accumulated sufficient substitutions to establish distinct genetic lineages for each of their genes. Their H1 HA gene, for example, is presently considered as a sublineage or clade of lineage 1C (Fig. 1). The genes of the present pandemic (H1N1) 2009 virus have the potential to lead to new genetic lineages. Among the 16 HA types at least 69 genetic lineages were described; the nine NA types comprise altogether 46 lineages and each of the internal segment has 7–11 lineages (Lu et al. 2007).

In addition to host species barriers, geographic isolation can induce the development of genetic lineages. As a result of different flyways of migratory birds, American and Eurasian lineages of influenza virus genes evolved (Olsen et al. 2006). As the evolution of such lineages is promoted by isolation rather than host-specific barriers, trans-species infections are not uncommon (Krauss et al. 2007; Wallensten et al. 2005; zu Dohna et al. 2009). They occur frequently in

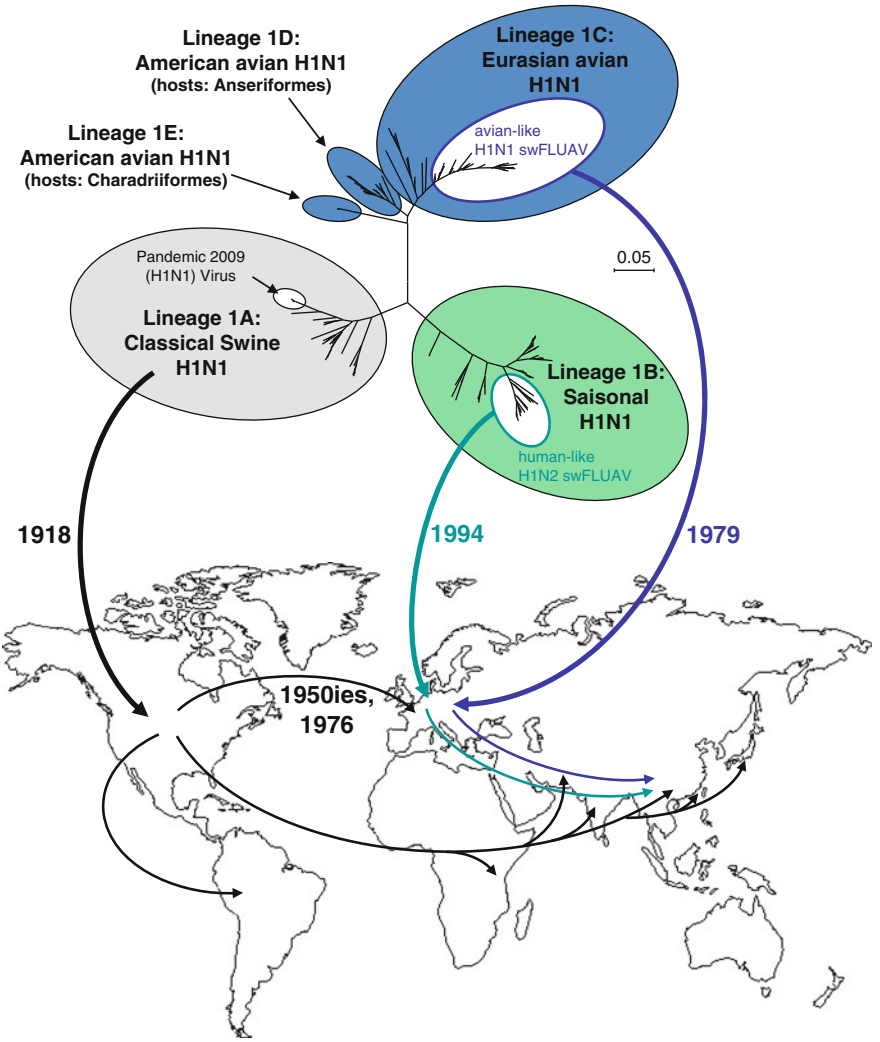


Fig. 1 Genetic lineages and sublineages of the HAH1 gene and their geographic distribution. Adaptation to host species and geographic isolation lead to the evolution of five HAH1 lineages (*top*). Genetic data suggest that the seasonal H1N1 viruses of humans emerged around 1918 from an American avian ancestor, either as a whole or as a reassortant (Anhlan et al. 2011), and spread worldwide (pandemic of 1918, not indicated). Classical swine H1N1 viruses also emerged around that time in the USA, probably after zoonotic infections of pigs. In several waves classical swine viruses were translocated to South America, numerous Asian countries, East Africa, and Europe. In Europe, they vanished after appearance of the avian-like H1N1 swine viruses in Belgium and Germany in 1979. Human-like H1N2 swine influenza viruses emerged in 1994 in the UK and spread to the European continent. After 2000, avian-like H1N1 and human-like H1N2 arrived in Asia where they co-circulate with the classical swine strains. Numerous reassortants indicate a dynamic influenza activity in Asia

overlapping breeding grounds, for example around the Arctic Beringia Sea (Wahlgren et al. 2008) or after translocation of infected birds (Makarova et al. 1999). Emergence of new lineages or subtypes may lead to the extinction of previously circulating types, a phenomenon that was repeatedly observed but cannot be sufficiently explained yet. For example, seasonal H1N1 viruses were superseded by pandemic H2N2 viruses in 1957. Likewise, circulating classical swine H1N1 viruses were replaced by avian-like swine H1N1 viruses after 1979 in Europe.

Despite the generation of thousands of sequence entries in the GenBank in recent years, our present understanding of the dynamics of the influenza virus epizootiology of birds and non-human mammals is still fragmentary.

5 The Disease

Swine influenza was originally described as a disease of autumn and early winter which occurred in annual epizootics (Shope 1931b). In many regions with dense pig populations the disease became enzootic and nowadays infections occur all year-round. The main symptoms of swine influenza are sudden onset of the disease, fever, anorexia, coughing, nasal discharge, sneezing, dyspnoea, exhaustion, and apathy. In general, infections with the virus cause a mild disease with a benign outcome. The morbidity within an affected herd is high (up to 100 %); the mortality is low but depends on the virus strain and other factors such as mixed infections. Usually the disease lasts 2–6 days and in most cases animals completely recover. Affected pigs develop an acute bronchitis with swollen mucosa, abundant mucus, hyperemia, and enlarged local lymph nodes. Inflammation surrounds bronchi and bronchioles. Sometimes secondary bacterial lobular pneumonia exacerbates the disease and may lead to death. Koen (1919) estimated influenza-associated mortality with "*1 per cent, at any rate less than 2 per cent*"; Shope with 1–4 % (Koen 1919; Shope 1931b).

In Europe, swine influenza is caused by three virus subtypes that are genetically distinct from the classical swine H1N1 viruses. In regions with enzootic persistence, the clinical signs are less marked and the virus circulates throughout the year. The avian-like swine H1N1 viruses generally induce less severe symptoms than human-like swine H3N2 viruses and natural H1N1 infections are sometimes unrecognized. The European H1N2 strains differ regarding their virulence. For example, the German strains which appeared in 2000 are more virulent than the Belgian strains. Changes in epizootiology may have several reasons. One reason is that swine husbandry practices changed in the past decades. A short fattening period of only 6 months leads to a rapid turnover of the swine population in a herd which requires purchase of piglets from various suppliers. This increases the chance of a virus to infect naive pigs and accomplishes gathering of influenza viruses from several distinct sources. Less marked clinical signs of enzootic viruses, varying levels of maternal antibodies, and preexisting immunities of older

pigs constitute selection pressures which are at the molecular level yet undefined and prepare the ground for the reassortment of novel virus combinations.

6 European Swine Influenza Viruses

There is evidence that influenza viruses have been introduced several times into European pigs. Stable infection chains, however, were first established in the 1970s. Prior to this, seasonal and classical swine influenza viruses have been detected in serological studies and occasional virus isolations. In this review we distinguish between zoonotic viruses of human and avian origin isolated from pigs on the one hand and the "*human-like*" and "*avian-like*" viruses on the other hand. Whereas the former viruses occur occasionally and become extinct after a few replication cycles, the latter viruses have defined genetic settings which developed distinct clades in phylogenetic trees. Such clades indicate stable infection chains over many virus generations. Moreover, these viruses exhibit evolutionary changes as a result of genetic drift and/or reassortment events which will be reviewed here. Previous authors (e.g., Brown 2000; Kuntz-Simon and Madec 2009) used the terms "*human-like*" and "*avian-like*" primarily to indicate the previous host.

6.1 Early Descriptions of Swine Influenza in Europe

Several early reports describe the influenza of pigs in Europe. Soon after the first isolation of swine influenza virus by Richard E. Shope, K. Köbe published the isolation of the etiologic agent of "enzootic pneumonia" of piglets, a condition that he named "Ferkelgrippe" (Köbe 1933). Köbe had observed that the histopathological lesions of the lungs were like "American swine influenza", but "Ferkelgrippe" in Germany showed enzootic rather than epizootic transmission and occurred in piglets younger than those of Shope's experiments. In analogy to Shope's work on swine influenza, Köbe believed that "Ferkelgrippe" was the result of a mixed infection; experimental pneumonia was induced only after coinfection of piglets with a filtrable virus that he had isolated from dispersed lung tissue of affected pigs and a bacterium that he designated *Bacterium influenzae suis*. The virus alone induced only mild symptoms and was distinct from the classical swine fever virus (hog cholera virus). It is unknown whether Köbe tried to propagate the virus in ferrets or mice. Unfortunately, the virus became lost in the past decades. Köbe's mentor Otto Waldmann confirmed the findings of his associate and commented that the observed differences in age incidence could be due to different husbandry practices in Germany and the USA (Waldmann 1933). Shortly thereafter, Gerhard Elkeles in Berlin, Germany, infected 2–6-week-old piglets with human influenza virus and could induce a mild flu-like disease in pigs; experimental coinfection of piglets with the human virus and either human or

porcine strains of *Haemophilus influenzae* resulted in a more severe disease (Elkeles 1934). These were the first experiments demonstrating the susceptibility of pigs to human influenza virus strains. Later they were confirmed by Shope and Francis (1936), but these authors used older pigs (6–14 weeks). With regard to the findings of Köbe and Elkeles, they discussed a different natural susceptibility of European and American pigs. Further work was published by Lamont (1938) and Blakemore and Gledhill (1941) who described outbreaks of swine influenza in Northern Ireland and England. Interestingly, Blakemore and Gledhill (1941) observed one outbreak on an Essex farm with cases of chronic disease (for 8 weeks), and—like O. Waldmann—concluded that husbandry conditions may have an influence on the course of the disease. Both Lamont and Blakemore handed over tissue specimens of several outbreaks to R. E. Glover in Cambridge who together with C. H. Andrewes succeeded to isolate three influenza virus strains after serial passages in ferrets and mice (Glover 1941). Serological characterization revealed that these isolates differed from Shope's swine influenza virus but resembled the human strains. Later, three of Glover's virus strains of that time were (partially) sequenced and were shown to cluster with A/WS/1933 and with A/Puerto Rico/8/1934 (Gorman et al. 1991; Neumeier and Meier-Ewert 1992; Neumeier et al. 1994; Yoshioka et al. 1994).

There is no hard evidence that classical swine influenza virus entered Europe in the 1930s or 1940s. European strains of classical swine influenza virus were first isolated in the 1950s in the former Czechoslovakia (Harnach et al. 1950). A serological survey conducted in 1957 by the W.H.O. revealed antibodies in pigs to classical swine H1N1 in Czechoslovakia and Germany (Kaplan and Payne 1959). After this episode, classical swine viruses disappeared in the 1960s in Europe, but were reintroduced in 1976 in Italy (Nardelli et al. 1978). These viruses spread to several European countries, including Belgium (Biront et al. 1980; Vandeputte et al. 1980), Germany (Sinnecker et al. 1983), France (Gourreau et al. 1980), England (Roberts et al. 1987), and Sweden (Martinsson et al. 1983). After the emergence of avian-like H1N1 swine viruses they disappeared again. The last European strain of classical swine H1N1 was isolated in 1993 in England soon after the first detection of avian-like H1N1 on the British Isles (Brown et al. 1997b).

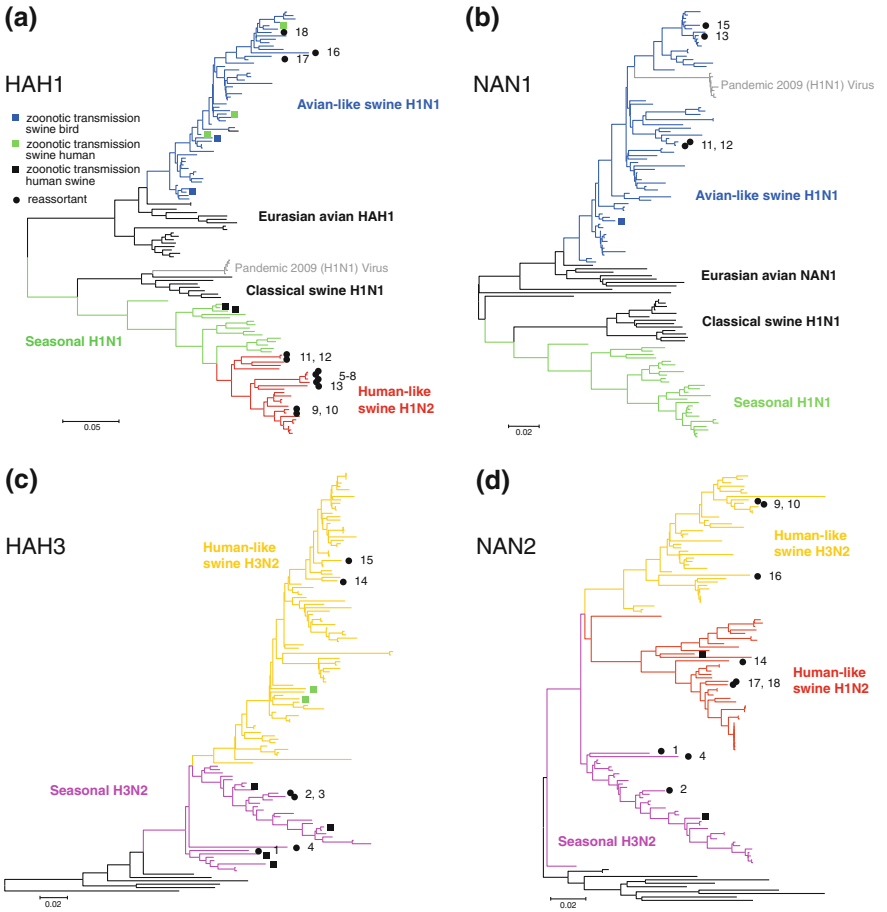
6.2 *Stable Establishment of Influenza Viruses in European Pigs: Avian-Like Swine H1N1*

A distinct sublineage of European H1N1 swine influenza viruses emerged in January 1979 in Belgium (Pensaert et al. 1981). These viruses differed serologically from classical swine viruses but showed relationship to avian viruses. Some virulent strains induced clinical symptoms which were typical for swine influenza. In winter 1979/80, similar viruses appeared in Germany and France (Witte et al. 1981; Ottis et al. 1981; Gourreau et al. 1981). Retrospective serological analyses

revealed that the majority of infections were asymptomatic. The molecular characterization revealed that all segments derived from an avian H1N1 influenza virus (Scholtissek et al. 1983). The phylogenetic comparison (Fig. 2a, b) demonstrates that the hemagglutinin of the swine viruses is most closely related to virus isolates from German ducks (A/duck/Bavaria/1/1977, A/duck/Bavaria/2/1977) which were the first avian H1N1 viruses detected in Europe (Ottis and Bachmann 1980). The hemagglutinin of the so-called "avian-like" swine H1N1 viruses shows a considerable cross-reaction with the classical swine H1N1; therefore, one of the commercially available vaccines (Gripovac™) for pigs includes A/New Jersey/8/1976 (H1N1). Further characterization revealed the avian origin of all segments (Schultz et al. 1991; Castrucci et al. 1993; Campitelli et al. 1997; Brown et al. 1997b). In very short time the avian-like H1N1 swine viruses established a stable infection chain and spread to all major swine-producing countries in Europe. They succeeded to replace the previous circulating classical swine strains. After 30 years of circulation, the avian-like swine H1N1 are endemic in the major pig-producing European countries. However, the seroprevalence varies considerably. In 2002/2003 it was highest in Belgium and Germany (80.8, 70.8 %); prevalence was lower in Italy and Spain; (46.4, 38.5 %) and low in the Czech Republic, Ireland, and Poland (>18 %) (Van Reeth et al. 2008). A more recent study conducted as a cross-sectional survey in Spain in 2008–2009 revealed a striking increase in the H1N1 seroprevalence (Simon-Grifé et al. 2010). Likewise, a German study indicates a similar annual variability suggesting fluctuations in the prevalence of swine influenza viruses over time (R. Dürrwald, personal communication).

6.3 Emergence of Human-Like H3N2 in European Pigs

The first "human-like" swine H3N2 virus emerged in Germany in 1982 (Schrader and Süss 2004). The HA and NA surface proteins of strain A/swine/Potsdam/35/1982 were derived from an A/Port Chalmers/1/1973-like seasonal H3N2 virus (Fig. 2c, d), whereas an avian-like H1N1 swine virus served as donor for the internal segments (M-segment: Schmidtke et al. 2006; Krumbholz et al. 2009; PB1-segment: Zell et al. 2007; PB2, PA, NP, NS segments: R. Zell unpublished). This virus disappeared soon. Another virus with a very similar genetic composition reemerged in 1984 and achieved to establish a persistent infection chain. The viruses were designated as "human-like swine H3N2" (Fig. 2c, d) due to their antigenic similarity to human H3N2. They spread rapidly in the European pig population. Epizootics were reported in Belgium (Haesebrouck et al. 1985; Haesebrouck and Pensaert 1988), France (Madec et al. 1984), and Germany (Zhang et al. 1989), Italy (Castrucci et al. 1993), the Netherlands (Loeffen et al. 1999), and Spain (Castro et al. 1988; Yus et al. 1992). The molecular analysis of these viruses revealed avian-like internal genes and human A/Port Chalmers/1/1973-like HA and NA genes (Campitelli et al. 1997; Marozin et al. 2002), but this



(e)

		PB2	PB1	PA	HA	NP	NA	M	NS
	Seasonal (human) H1N1	A	A	B	1B	A	1A	B	1A
	Seasonal (human) H3N2	A	D	B	3A	A	2A	B	1A
	avian-like swine H1N1: A/swine/Arnsberg/6554/1979-like	F	G	I	1C	F	1F	F	1E
	human-like swine H3N2: A/swine/Gent/1/1984-like	F	G	I	3A	F	2A	F	1E
	human-like swine H1N2: A/swine/Scotland/410440/1994-like	F	G	I	1B	F	2A	F	1E
1	A/swine/Potsdam/35/1982 (H3N2)	F	G	I	3A	F	2A	F	1E
2	A/swine/Karrenzien/2/1987 (H3N2)	F	G	I	3A	F	2A	F	1E
3	A/swine/Jena/3765/1989 (H3N2)	/	/	/	3A	/	/	/	/
4	A/swine/Leipzig/145/1992 (H3N2)	F	G	I	3A	F	2A	F	1E
5	A/swine/Italy/19447/2006 (H1N2)	/	/	/	1B	/	2A	/	/
6	A/swine/Italy/19498/2006 (H1N2)	/	/	/	1B	/	2A	/	/
7	A/swine/Italy/23255/ (H1N2)	/	/	/	1B	/	2A	/	/
8	A/swine/Italy/30073-5/2006 (H1N2)	/	/	/	1B	/	2A	/	/
9	A/swine/Ötitingen/IDT4735/2005 (H1N2)	F	G	I	1B	F	2A	F	1E
10	A/swine/Cloppenburg/IDT4777/2005 (H1N2)	F	G	I	1B	F	2A	F	1E
11	A/swine/Cotes d'Armor/60293/2001 (H1N1)	F	G	I	1B	F	1F	F	1E
12	A/swine/Cotes d'Armor/98574/2001 (H1N1)	F	G	I	1B	F	1F	F	1E
13	A/swine/Cotes d'Armor/0190/2006 (H1N1)	F	G	I	1B	F	1F	F	1E
14	A/swine/Nordkirchen/IDT1993/2003 (H3N2)	F	G	I	3A	F	2A	F	1E
15	A/swine/Italy/66945/2006 (H3N1)	F	G	I	3A	F	1F	F	1E
16	A/swine/Sweden/1021/2009 (H1N2)	F	G	I	1C	F	2A	F	1E
17	A/swine/Italy/2064/1999 (H1N2)	F	G	I	1C	F	2A	F	1E
18	A/swine/Germany/SEk1178/2000 (H1N2)	/	/	/	1C	F	2A	F	1E

Fig. 2 Phylogenetic trees. 125 representative sequences were aligned and used to infer the evolutionary relationships using the neighbor-joining method. Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007). Avian-like swine influenza virus sequences are indicated in *blue*, human-like H3N2 in *ochre*, human-like H1N2 in *red*, seasonal H1N1 in *green*, and seasonal H3N2 in *pink*. Strain designations were omitted for clarity. **a** HAH1 sequences, **b** NAN1 sequences, **c** HAH3 sequences, **d** NAN2 sequences, **e** genetic composition of seasonal H1N1, H3N2 lineages, the prevalent European swine H1N1, H1N2, and H3N2 sublineages and 18 reassortants. Genotyping was done according to Lu et al. 2007

parental human H3N2 virus was clearly distinct from that of the previous German strain (Fig. 2c, d; see also Schrader and Süss 2004). In most European countries the seroprevalence in 2002/2003 of the human-like swine H3N2 is lower than that of avian-like H1N1. Human-like swine H3N2 is (almost) absent in Poland and the Czech Republic, very low in Ireland (4.2 %), and below 60 % in Belgium and Germany. Only in Italy and Spain H3N2 prevalences are as high as H1N1 prevalences (Van Reeth et al. 2008; Simon-Grifé et al. 2010).

6.4 Emergence of Human-Like H1N2 in European Pigs

Swine H1N2 viruses that became prevalent in Europe were first isolated in Great Britain in 1994 (Brown et al. 1995; 1998). Available sequence data indicate that these H1N2 viruses resulted from repeated reassortment events involving a seasonal A/Chile/1/1983-like H1N1 virus (donor of HA) and a seasonal H3N2 virus (donor of NA) (Fig. 2a, d). Apparently, human H3N2 viruses circulated in pigs unrecognized for several years, as one member of this clade was already isolated in 1991 (A/swine/UK/119404/1991) (compare Zell et al. 2008b, therein Fig. 1b). Since the avian-like swine H1N1, but no human-like H3N2 viruses, circulated among British pig during that time, it has to be concluded that the former viruses were the donor of the internal segments. Three years later, the human-like swine H1N2 viruses spread to the European mainland: France (1997), Italy (1998), Belgium (1999), and Germany (2000) (Marozin et al. 2002; Van Reeth et al. 2000; Schrader and Süss 2003). In 2002/2003, the seroprevalences of H1N2 in Belgium and Spain exceeded that of human-like H3N2; it was low in Germany (32.1 %) and Italy (13.8 %) and very low in the Czech Republic (3 %) and Ireland (0.6 %) (Van Reeth et al. 2008).

The evolution of the three prevalent sublineages of the European swine influenza viruses is schematically depicted in Fig. 3.

6.5 Other Reassortant Swine Influenza Viruses Isolated in Europe

Sooner or later co-circulation of two or more influenza virus types within a population leads to reassortant viruses, but such reassortants may have little chance to replace either parent virus. The three prevalent European swine influenza viruses gave rise to three groups of reassortants. The first group comprises reassortants of seasonal human H3N2 and swine influenza viruses (Fig. 4). The strains A/swine/Potsdam/35/1982, A/swine/Karrenzien/2/1987, and A/swine/Leipzig/145/1992 (Schrader and Süss 2004) are examples of swine H3N2 viruses which emerged independently of each other in Germany. They have the six internal gene segments

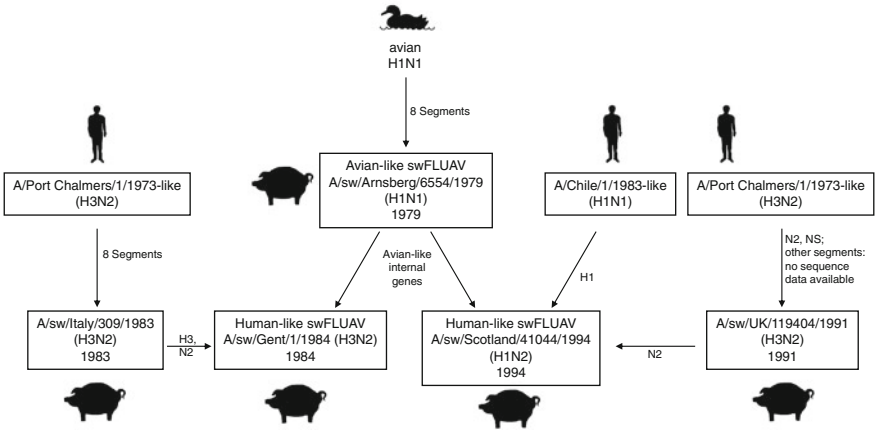


Fig. 3 Evolution of three prevalent sublineages of European swine influenza viruses

of avian-like H1N1 swine viruses and human HA and NA genes. These genes, however, branch independently of A/swine/Gent/1/1984-like viruses in phylogenetic trees (compare Fig. 2c, d) and are evidence of repeated 2 + 6 reassortments in pigs. Although only partial sequence data are available, further strains e.g., A/swine/Jena/3765/1989 (H3N2), A/swine/Leipzig/663/1992 (H3N2), and A/swine/Leipzig/318/1993 (H3N2), belong to this group and indicate that such reassortants may have circulated for 2–3 years. The second group of reassortants emerged in Italy. The preliminary characterization revealed a 7 + 1 reassortment between human H3N2 and swine H1N2 influenza viruses. These viruses have a neuraminidase gene of seasonal H3N2 viruses and seven segments (HA, internal genes) of human-like swine H1N2 viruses (Chiapponi et al. 2007). They circulated in Italy between 2003 and 2006.

The third group comprises reassortants between the prevalent sublineages of European swine influenza viruses. The three sublineages allow six HA/NA combinations and all of them have been detected in recent years. The compilation of Fig. 4 illustrates that several of these reassortments occurred repeatedly at different places and times: Some of the reassortants were published (Gourreau et al. 1994; Balint et al. 2009; Zell et al. 2008a, b), for others only preliminary reports are available (Chiapponi et al. 2007; Franck et al. 2007; Hjulsager et al. 2006). Such reassortants do not constitute antigenic shift mutants and failed to establish persistent stable infection chains yet.

Another rather unusual reassortant was isolated from pigs in England (Brown et al. 1994). The strain A/swine/England/191974/1992 (H1N7) was reported to comprise six segments of a human H1N1 virus (PB2, PB1, PA, HA, NP, NS) and the NA and M segments of an equine H7N7 virus (Brown et al. 1997a). Sequence data of the HA, NP, NA, and M segments are available in the GenBank. Although this virus represents an interesting reassortment, it has to be considered with some caution as the NA and M genes have a striking similarity to A/equine/Prague/1/

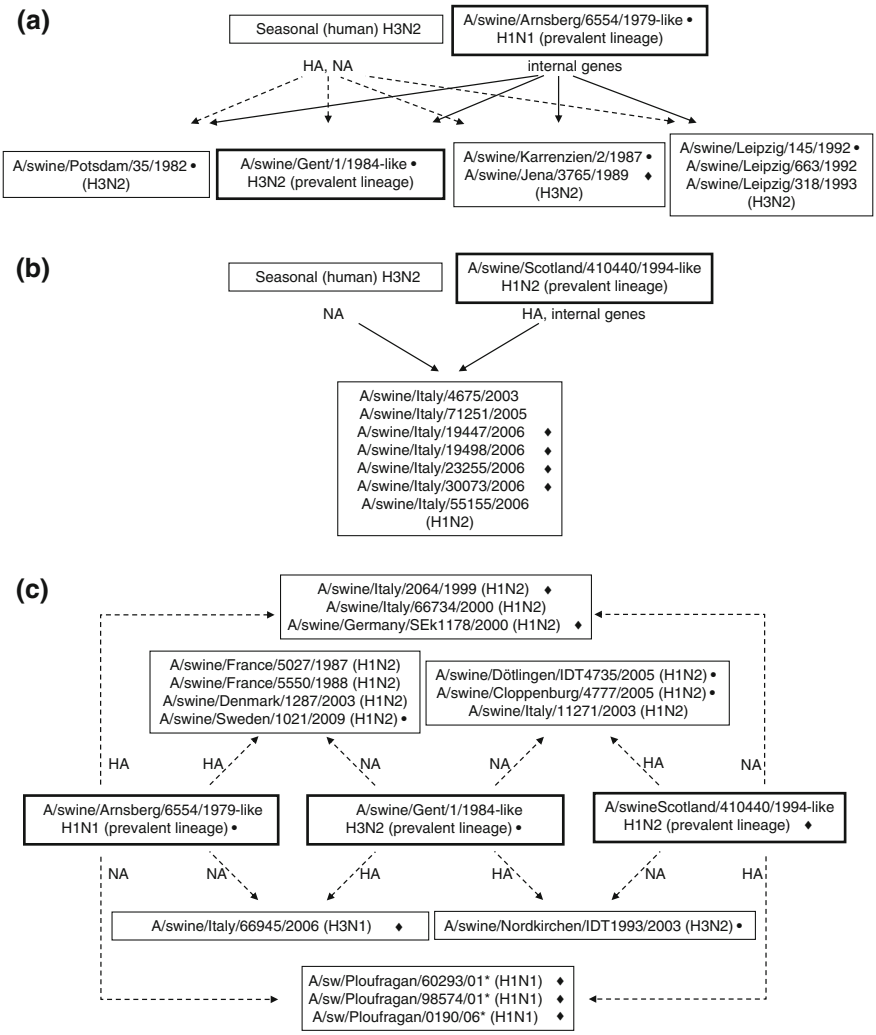


Fig. 4 Three groups of reassortant swine influenza viruses emerged in Europe. **a** 6 + 2 reassortants of avian-like swine H1N1 and human H3N2 influenza viruses. **b** 7 + 1 reassortants of human-like swine H1N2 and human H3N2 viruses. **c** Six different reassortants of the three prevalent European swine influenza virus lineages. The prevalent swine influenza virus lineages are boxed with bold lines. *Filled circles* (•) indicate the availability of complete sequence data in the GenBank, *filled diamonds* (◆) indicate partial sequence data. * Strains designations follow those of Franck et al. (2007); these strains were renamed when their sequences were deposited in the GenBank

1956 (H7N7). All other equine H7N7 sequences available from the GenBank (isolates of 1966–1977) show synonymous substitutions as a consequence of genetic drift and therefore differ significantly from A/equine/Prague/1/1956.

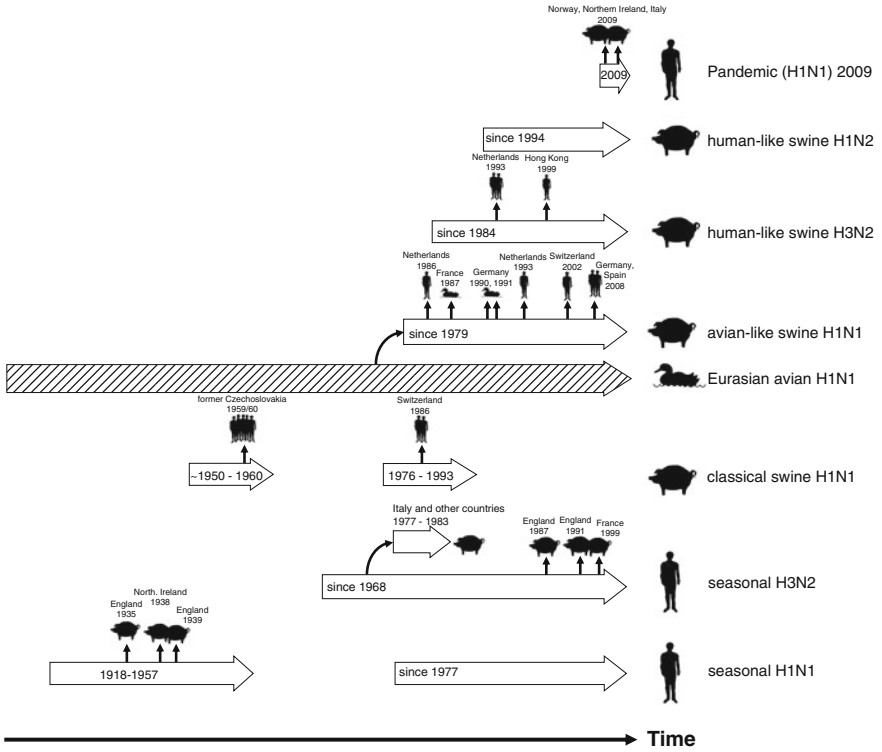


Fig. 5 Zoonotic infections of influenza viruses in Europe over time. Indicated are the relevant lineages of human, avian, swine influenza viruses in Europe and characteristic virus isolates

Equine H7N7 viruses disappeared around 1977 and it is quite astounding that an A/equine/Prague/1/1956-like virus should have persisted in an unknown reservoir for 36 years without accumulation of synonymous substitutions. Therefore, the biological significance of this reassortant should be scrutinized.

7 Zoonotic Infections

7.1 Human→Swine Infections

Human H1N1 influenza viruses have only a limited capacity to productively infect pigs (Hinshaw et al. 1978). However, there are several swine isolates of human origin from the 1930s which were isolated from clinically ill pigs (Lamont 1938; Blakemore and Gledhill 1941). Serological similarity of these strains to contemporary human strains was already observed by Glover (1941). Evidence for zoonotic infections of pigs by human H1N1 was also presented by Shope (1938). Thereafter,

human–swine infections with H1N1 have not been documented in Europe, especially not after reemergence of H1N1 in 1977 (Fig. 5). However, there is indirect evidence that such infections may have occurred: (i) the emergence of H1N2 reassortants in swine in Europe (Brown et al. 1998; Marozin et al. 2002), and (ii) the observed antibody prevalence to human H1N1 in pig sera (Aymard et al. 1980). In Japan and China, several studies demonstrate the transmission of seasonal H1N1 to pigs as shown by virus isolation and seroprevalence studies (Goto et al. 1988; Katsuda et al. 1995; Nerome et al. 1982; Yu et al. 2007).

The human H2N2 viruses have never been isolated from pigs after natural infection, although there is one study that showed antibodies against H2N2 in four pigs in the former Czechoslovakia (Kaplan and Payne 1959). In principle, pigs are susceptible to these viruses as experimental infection of pigs with A/Singapore/1/1957 (H2N2) was successful (Patocka et al. 1958).

Seasonal H3N2 viruses were frequently detected in pigs in Europe and elsewhere. This is documented in several serological studies from Germany, the UK, France, Romania, and the Czech Republic (Sandow and Wildfuhr 1970; Harkness et al. 1972; Popovici et al. 1972; Aymard et al. 1980; Tumova et al. 1980; Pospisil et al. 2001). Occasionally, human H3N2 viruses were isolated from pigs (A/swine/England/163266/1987, A/swine/United Kingdom/119404/1991; Brown et al. 1998). In Italy, A/England/42/1972-like H3N2 viruses persisted from 1977–1983 in pigs (Ottis et al. 1982; Castrucci et al. 1993). Moreover, zoonotic infections with seasonal H3N2 gave rise to numerous reassortant viruses: the European human-like swine H3N2 lineage and the American tripple reassortants are the most prominent representatives (Castrucci et al. 1993; Olsen 2002; Karasin et al. 2000c). Figure 5 shows a compilation of zoonotic influenza virus infections in Europe.

The pandemic (H1N1) 2009 virus was repeatedly transmitted to pigs, first in Canada (Pasma and Joseph 2010), later in Norway, Northern Ireland, Italy (Hofshagen et al. 2009; Welsh et al. 2010; Moreno et al. 2010), and other European countries. The available reports indicate that this new reassortant induces a mild disease (Brookes et al. 2010; Itoh et al. 2009; Lange et al. 2009) and that many infections may be unrecognized. In addition, pandemic (H1N1) 2009 virus exhibits a significant cross-reaction to antibodies against avian-like swine H1N1 which impedes serological distinction (Kyriakis et al. 2010; Dürrwald et al. 2010). On the other hand, this cross-reactivity may hinder establishment of the pandemic virus in regions with high prevalence of avian-like swine H1N1.

7.2 Bird→Swine Infections

Although an initial bird→swine infection gave rise to the avian-like swine H1N1 sublineage, no discrete infections with avian influenza viruses have been documented in Europe. A similar observation was previously made in North America, when the genetic origin of 73 swine isolates (1976–1990) was investigated and no

entry of avian genes could be detected (Wright et al. 1992). Later studies, however, revealed several of such infections in Asia, America, and Africa (Guan et al. 1996; El-Sayed et al. 2010; Karasin et al. 2000a, b; 2004; Yu et al. 2007, 2008; Peiris et al. 2001; Lee et al. 2009).

7.3 Swine→Bird Infections

In the 1980s and early 1990s, at least three swine-origin strains were isolated from birds (Andral et al. 1985; Ludwig et al. 1994; Wood et al. 1997). Partial genetic analyses revealed a reintroduction of avian-like swine H1N1 viruses into turkey farms (Ludwig et al. 1994). Improved hygiene in poultry husbandry and advanced adaptation of the swine H1N1 to its pig host may explain the failure of virus isolation in recent years in Europe. Human-like swine H3N2 and H1N2 strains have not been isolated from birds yet. Absence of α-2,6-linked sialic acids in poultry may be the main reason for the inability of human-like H3N2 and H1N2 sublineages to replicate in birds. The chapter by Yassine and colleagues on "Interspecies transmission of Influenza A viruses between swine and poultry" in this book described these interspecies infections in more detail.

7.4 Swine→Wild Boar Infections

In principle, wild boars should be susceptible to influenza viruses of swine and avian origin and may serve as a reservoir for such viruses. Although they have contacts to feral birds, the possibility of a transmission of avian influenza viruses to feral pigs is only insufficiently investigated in Europe. However, several serological studies searched for antibodies to swine influenza viruses in wild boars (recently reviewed in Kuntz-Simon and Madec 2009). Antibodies to avian-like swine H1N1 influenza viruses in feral pigs were detectable in Spain, Poland, and Croatia but not in Slovenia, Russia, and Ukraine. Another recent study demonstrated antibodies to avian-like swine H1N1 and human-like swine H3N2 viruses in Germany (Kaden et al. 2008). Two virus isolates described in that study [A/wild boar/WS169/2006 (H3N2), A/wild boar/WS188/2006 (H3N2)] should be considered with caution. The sequences of both isolates are identical and the published sequences of five different gene segments (HA, NP, NA, M, NS) show a sequence identity of nearly 100 % to A/swine/Bakum/909/1993 (H3N2) which was used as a H3N2 control in this study. Since influenza viruses exhibit a genetic drift due to the accumulation of synonymous and non-synonymous substitutions, one would expect some genetic variation in the course of several hundred virus generations (1993–2006). The wild boar isolates obviously lack this genetic drift.

Table 2 Zoonotic infection

No.	Country	Years	No. of patients	Type	Designation	Reference
1	Former Czechoslovakia	1959	6	Classical swine H1N1	No information available	Kluska et al. (1960)
2	Switzerland	1986	2	Avian-like swine H1N1	No information available	de Jong et al. (1986)
3	The Netherlands	1986	1	Avian-like swine H1N1	A/Netherlands/386/1986	de Jong et al. (1986); Rimmelzwaan et al. (2001)
4	The Netherlands	1993	1	Avian-like swine H1N1	A/Netherlands/477/1993	Rimmelzwaan et al. (2001)
5	The Netherlands	1993	2	Human-like swine H3N2	A/Netherlands/5/1993, A/Netherlands/35/1993	Claas et al. (1994)
6	Hong Kong	1999	1	Human-like swine H3N2	A/Hong Kong/1774/1999	Gregory et al. (2001)
7	Switzerland	2002	1	Avian-like swine H1N1	A/Switzerland/8808/2002	Gregory et al. (2003)
8	Germany	2007	1	Avian-like swine H1N1	A/Niedersachsen/58/2007	Schweiger et al. (2008)
9	Spain	2008	1	Avian-like swine H1N1	A/Aragon/RR3218/2008	Adiego Sancha et al. (2009)

7.5 Swine→Human Infections

Swine-to-human transmissions of classical swine H1N1 influenza viruses were first observed in Czechoslovakia in the 1950s (Kluska et al. 1961). Since then, sporadic infections were repeatedly demonstrated by virus isolation in the United States, Europe, and the Asian part of the former Soviet Union (reviewed in Myers et al. 2007). Several incidents of human infection with the European avian-like H1N1 and human-like H3N2 swine influenza viruses have been reported so far (Table 2) (Adiego Sancho et al. 2009; Claas et al. 1994; de Jong et al. 1986; Gregory et al. 2001, 2003; Rimmelzwaan et al. 2001; Schweiger et al. 2008). Apparently, zoonotic infections with the European swine viruses cause a benign disease with mild flu-like symptoms, whereas infections with classical swine strains may lead to more serious symptoms—few fatalities after infections with the latter viruses were reported (Myers et al. 2007). Despite repeated isolation of swine influenza viruses from human specimens, the prevalence of zoonotic infections in Europe is largely obscure. Previous work demonstrated seropositivity of personnel having contact to diseased pigs (Aymard et al. 1980; Sinnecker et al. 1983). A recent study conducted in Thuringia, Germany, indicates that approximately 15 % of the investigated sera of occupationally exposed humans (pig farmers, slaughterers, veterinarians) exhibit antibodies to the European lineages of swine influenza viruses (Krumbholz et al. 2010).

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History of Swine Influenza Viruses in Asia

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Abstract The pig is one of the main hosts of influenza A viruses and plays important roles in shaping the current influenza ecology. The occurrence of the 2009 H1N1 pandemic influenza virus demonstrated that pigs could independently facilitate the genesis of a pandemic influenza strain. Genetic analyses revealed that this virus was derived by reassortment between at least two parent swine influenza viruses (SIV), from the northern American triple reassortant H1N2 (TR) and European avian-like H1N1 (EA) lineages. The movement of live pigs between different continents and subsequent virus establishment are preconditions for such a reassortment event to occur. Asia, especially China, has the largest human and pig populations in the world, and seems to be the only region frequently importing pigs from other continents. Virological surveillance revealed that not only classical swine H1N1 (CS), and human-origin H3N2 viruses circulated, but all of the EA, TR and their reassortant variants were introduced into and co-circulated in pigs in this region. Understanding the long-term evolution and history of SIV in Asia would provide insights into the emergence of influenza viruses with epidemic potential in swine and humans.

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Current Topics in Microbiology and Immunology (2013) 370: 57–68
DOI: 10.1007/82_2011_179

Published Online: 23 September 2011

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1 Introduction

Asia is home to the world's largest human population. With its rapid growth and increasing wealth, Asia has an escalating need and demand for more and better quality food sources. Farming practices in Asia have been greatly changed by industrialization and globalization in the quest for greater production. Importation of breeding pigs from the USA and Europe and the establishment of intensive pig-breeding farms have caused the population of pigs in Asia to soar. Now, approximately 60% of the world's pigs are in Asia and China alone is home to over 40% of the world's pigs (USDA/FAS).

Greatly increased swine numbers have led to enhanced opportunities for contact between pigs and humans and between pigs and the similarly enlarged poultry flocks and the wild birds of Asia. Interspecies transmission of influenza to and from swine has been frequently observed (Pensaert et al. 1981; Mohan et al. 1981; Claas et al. 1994; Guan et al. 1996) and pigs are regarded as a major intermediate host in the process of adapting avian viruses to mammalian hosts (Scholtissek et al. 1985; Scholtissek 1990). Highly pathogenic H5N1 and low pathogenic H9N2 avian influenza viruses have become enzootic in Asia and transmissions to humans and pigs have occurred (Webster et al. 2006; Peiris et al. 1999; Lin et al. 2000; Claas et al. 1998; Subbarao et al. 1998). The 2009 pandemic virus had its origins in currently circulating swine influenza viruses (Dawood et al. 2009; Garten et al. 2009; Smith et al. 2009b).

Both the economic consequences to food production and the threat to human health emphasize the importance of swine influenza. Monitoring the evolution and ecology of this virus is an essential task for human well-being. This is especially the case in Asia where the largest population of pigs in the world interacts with such a large human population.

2 Prevalence and Detection of Swine Influenza Viruses in Asia

H1N1 and H3N2 influenza viruses occur in pigs in Asia but the clinical picture of infection is less clear. Pig farming in China and much of Asia has been traditionally based on small family holdings and only in the last 20 years or so have large-scale farming operations come onstream. Consequently, relatively little surveillance has occurred and there is limited information about swine influenza in Asia in the wider scientific literature.

2.1 Classical Swine H1N1 Virus

The presence of classical swine H1N1 (CS) influenza in China probably dates back to the 1918–1919 pandemic when, in the wake of human infections, an outbreak of high mortality occurred in pigs in cities along the Chinese coast (Chun 1919; Kilbourne 2006). A similar disease pattern was observed in the USA with humans infected before pigs (Koen 1919; Reid and Taubenberger 2003). Findings from evolutionary studies revealed that classical swine H1N1 and 1918 pandemic H1N1 viruses shared a common ancestor or were highly closely related to each other (Smith et al. 2009a; Kanegae et al. 1994; Gorman et al. 1991). This historic interrelationship between human and porcine H1N1 viruses may be similar to the current situation with H1N1/2009 viruses. Since its emergence, the 2009 human pandemic H1N1 virus has been repeatedly transmitted from humans to pigs (Pereda et al. 2010; Weingartl et al. 2010; Vijaykrishna et al. 2010).

Although the CS virus was isolated and identified as early as 1930 in the USA (Shope 1931), it was first isolated in Asia in 1974 (swine/Hong Kong/1/74). Since the mid-1970s influenza surveillance of pigs conducted in Hong Kong and Japan has revealed that classical H1N1 viruses are widely distributed in many Asian regions and countries. Surveillance in Hong Kong from 1976 to 1980 took samples from pigs grown in Hong Kong and pigs imported from Mainland China, Taiwan and Singapore (Shortridge and Webster 1979). Regular isolation of classical swine viruses showed their continuing presence in apparently healthy pigs (Shortridge and Webster 1979; Yip 1976). During the same period of time, serological studies revealed that classical swine H1N1 viruses were also common in the pig populations of Japan (Arikawa et al. 1979; Nerome et al. 1982; Ogawa et al. 1983; Yamane et al. 1978).

Further surveillance in Hong Kong during 1993–1994 showed that classical H1N1 viruses were apparently the predominant influenza virus infecting pigs (Guan Y, unpublished). A clear epizootic occurred, with large numbers of viruses isolated in February, March and April of 1994. Little surveillance has been conducted in other countries of Asia. Classical H1N1 swine influenza viruses were reported to be isolated in Thailand in 1988 (Kupradinun et al. 1991) and Mainland China in 1991 (Guan et al. 1996), and this virus was also identified from pigs in

Korea (Lee et al. 2008; Song et al. 2007) and India (Das et al. 1981; Chatterjee et al. 1995).

Generally, classical H1N1 viruses were genetically stable and showed minor antigenic drift in Asian countries. However, a reassortant H1N2 virus, with its N2 segment from early human-like H3N2 viruses and its remaining segments from classical H1N1 viruses (Sugimura et al. 1980), caused a major outbreak in southern Japan from winter 1989 to spring 1990. Affected pigs had a typical influenza illness and most swine tested possessed corresponding antibodies (Ouchi et al. 1996). Similar reassortant viruses were also detected in Hong Kong from pigs imported from China during 1999 to 2004 (Vijaykrishna et al. 2011).

Phylogenetic analyses of large data sets of swine influenza viruses reveal that the classical H1N1 viruses isolated in Hong Kong do not form a single monophyletic group. They are interspersed with North American CS viruses, indicating multiple introductions of CS into Asian countries from the USA (Vijaykrishna et al. 2011). However, it is hard to believe that the classical H1N1 viruses from different Asian countries were all introduced from the USA via importation of pigs from there. CS viruses might have evolved from the 1918 pandemic H1N1 virus as it became able to persist in pigs. Supposedly, then, this virus should have existed in all regions where pigs were available. However, the effect of long-term vaccination practices in the USA might induce an evolutionary advantage in CS viruses from that region, allowing them to replace previously existing CS strains. This hypothesis could be possibly tested again as the 2009 pandemic H1N1 virus and its variants become established in pigs.

2.2 H3N2 Human-like Influenza Viruses

H3N2 swine viruses appear to be the result of multiple transmissions of viruses from humans to pigs. They were first isolated in Asia from pigs in Taiwan soon after the Hong Kong pandemic (Kundin 1970). During surveillance in Hong Kong from 1976 to 1982, and from 1998 to the present, contemporary variants of H3N2 human-like viruses and antibodies to them were regularly isolated or detected in pigs from Asian countries (Shortridge and Webster 1979; Vijaykrishna et al. 2011; Shortridge et al. 1977; Webster et al. 1977). Based on our long-term surveillance, almost all of the major human H3N2 variants could be introduced into the pig population (Vijaykrishna et al. 2011). Some of these variants might remain in somewhat genetically dormant states, akin to evolutionary stasis, in pigs even many years after their counterparts had disappeared from humans (Shortridge et al. 1977).

The first two major H3N2 variants were A/Port Chalmers/1/73 (PC) and A/Victoria/3/75 (Vic) (Shortridge and Webster 1979; Shortridge et al. 1977, 1979). Interspecies transmission to pigs of these two viruses occurred in many Asian countries, including China, Korea and Japan (Shortridge and Webster 1979; Shortridge et al. 1977, 1979; Song et al. 2003; Jung and Song 2007; Yamane et al.

1979; Nerome et al. 1981; Arikawa et al. 1982). In Europe, PC-like H3N2 viruses reassorted with European avian (EA)-like H1N1 viruses to generate an H3N2 virus with PC-like surface genes and EA H1N1 internal genes (Campitelli et al. 1997; Castrucci et al. 1993). This H3N2 reassortant has been maintained in European countries since then and was introduced into Asian countries in the late 1990s (Gregory et al. 2001) (see below). The third major human H3N2 variant to cause zoonotic outbreaks in pigs was A/Sydney/05/97 (Syd). Introduction of this variant into pigs occurred, at least, in China and the USA (Peiris et al. 2001; Yu et al. 2008; Zhou et al. 1999). In China, the virus kept the entire human-like particle, while in the North America it further reassorted with CS and avian viruses to generate double and triple reassortant viruses (TR, H3N2, H1N2 and H1N1 subtypes) (Zhou et al. 1999; Karasin et al. 2006).

Since 2005, although H3N2 viruses have failed to be detected in the pig population under our surveillance program in Hong Kong (Vijaykrishna et al. 2011), contemporary and early human H3N2 variants were still isolated from pigs in the wider region (Hause et al. 2010; Lekcharoensuk et al. 2010; Kyriakis et al. 2011). Except for the major variants mentioned above, most human-like H3N2 variants seem to be transient and to have difficulty in becoming established in pigs as none have formed an independent group or sublineage in the evolutionary trees. Thus, it is likely that human H3N2 variants were regularly introduced into pigs, but most were prevalent at a low level within a small geographic location, and failed to become established.

2.3 European H3N2 Reassortant Viruses

In 1999, an H3N2 reassortant swine virus (represented by swine/Hong Kong/5212/99) was isolated in Hong Kong from pigs imported from southern China. This virus was antigenically and genetically distinct from the human-like H3N2 viruses then circulating in pigs. Genetic analyses revealed that this virus had PC-like surface genes and EA-like internal genes (Vijaykrishna et al. 2011). It was closely related phylogenetically to the European H3N2 reassortant viruses that were generated in the mid-1980s and have circulated in Europe since then (Claas et al. 1994; Campitelli et al. 1997; Castrucci et al. 1993). This virus caused a human infection case in Hong Kong (Gregory et al. 2001).

These findings provide a typical example of the direct introduction of swine influenza virus from Europe to Asia, very likely via pig movement. To largely increase its pig population and production, China started to import breeding pigs from European countries, such as Denmark (e.g. the DanBred organization) and set up breeding pig farms in the Zhujiang delta region in Guangdong since the mid-1990s. Unfortunately, the swine influenza virus was not included on Chinese warrant agent lists before 2009. This European H3N2 reassortant virus seemed to only circulate in Guangdong province in southern China and has not been reported from any other places in Asia.

2.4 European Avian-like H1N1

The first detection of EA viruses outside European countries occurred in early 2001 in Hong Kong. The virus (swine/Hong Kong/8512/01) (Smith et al. 2009; Vijaykrishna et al. 2011) was isolated from pigs imported from southern China. Since then, this virus has co-circulated with other swine influenza viruses, including CS, H3N2 and, later, American triple reassortant H1N2 (see below) viruses in this region and gradually became predominant from 2006. The replacement of CS viruses with EA viruses in pigs in the field took 4 to 5 years, similar to what happened in Europe after the EA lineage became established in pigs in the mid 1980s (Brown 2000). Phylogenetic analyses showed that the EA viruses isolated from pigs in China form a monophyletic group, suggesting a single introduction of this virus (Vijaykrishna et al. 2011).

H1N1 reassortants between CS and EA viruses were also detected in 2001 (Fig. 1), very likely occurring at the beginning of the introduction of EA viruses. Genetic analyses based on publicly available data showed that the EA virus was also introduced into pigs in Thailand around 2000 (Takemae et al. 2008). This virus also reassorted with CS viruses in pigs in this country (Takemae et al. 2008). Currently, EA viruses or its variants have become a major swine influenza lineage prevailing in the region. Whether the predominance of EA H1N1 viruses in the field is directly affecting the prevalence of H3N2 viruses is still unknown.

2.5 American Triple Reassortant Virus

In the reassortment event of 1998, both H3N2 and H1N2 triple reassortant viruses were generated in North America (Zhou et al. 1999; Karasin et al. 2006). Since 2002 American triple reassortant H1N2 viruses were regularly isolated from pigs in our surveillance program in China (Fig. 1). This virus has prevailed since then at generally low levels in pigs but was more prevalent during 2004 (Vijaykrishna et al. 2011). Our surveillance program suggests that triple reassortant viruses were introduced to China on several occasions (Vijaykrishna et al. 2011). From 2004, both H1N2 and H3N2 triple reassortant viruses have been isolated from pigs in Korea (Jung and Chae 2004; Pascua et al. 2008) Given that different subtypes with gene segments falling into polyphyletic groups were found in these two countries, separate introductions of the virus from the USA appear to have occurred.

2.6 Reassortant Viruses Between EA and TR

The 2009 pandemic H1N1 virus was derived by reassortment from several swine influenza viruses, which might include EA viruses (for the NA and M segments), European H3N2 reassortant viruses (for the M segment) and TR viruses (for the

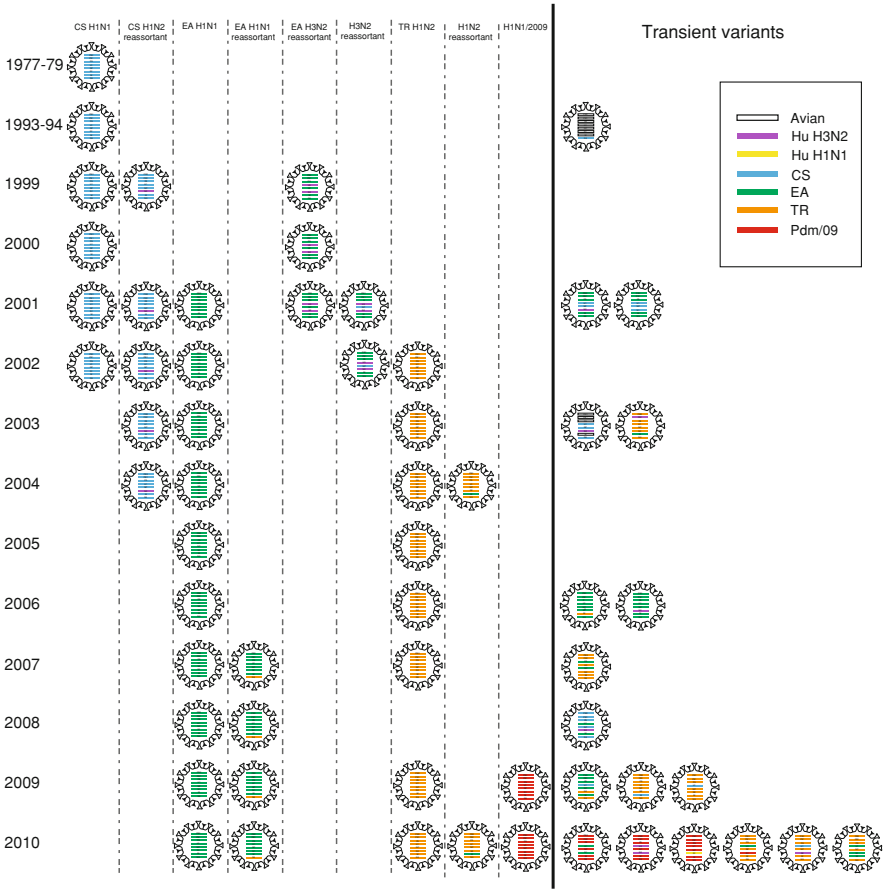

Fig. 1 Timeline of the genotype of viruses identified in surveillance program in China. Genotypes observed over at least two years are shown to the left. Segments are ordered by size from top to bottom for each genotype. The lineage of origin of each segment is indicated by *color*: *white* avian origin, *purple* human seasonal H3N2, *yellow* human seasonal H1N1, *blue* classical swine (CS), *green* European avian-like (EA), *orange* American triple reassortant (TR), *red* pandemic H1N1 2009 (Pdm/09)

remaining six segments) (Garten et al. 2009; Smith et al. 2009b). Thus, co-circulation of EA and TR viruses would appear to be essential for the genesis of this pandemic virus. Based on our surveillance findings and publicly available data, co-circulation of EA and TR viruses were observed in China from 2003 onwards (Fig. 1). However, EA-like viruses were never reported from America and TR-like viruses were also not reported from European countries. It is plausible, therefore, that the 2009 pandemic H1N1 virus could have been generated within the pigs of an Asian country (most likely China). Indeed, reassortment

events between EA and TR viruses are not rare, and reassortant swine viruses with differing genotypes have been recognized in the field (Fig. 1). Reassortants between EA and TR viruses, which are the most closely related to the 2009 H1N1 pandemic virus (by having seven gene segments from the same lineages), were isolated in southern China on four sampling occasions (two before and two after the pandemic). However, all these reassortants had different evolutionary pathways, and almost all reassortants between EA and TR viruses appear to be transient. The only exception was a reassortant which had seven segments from the EA lineage and the NS segment from the TR lineage and has become established in the field and may eventually predominant in pigs in China (Vijaykrishna et al. 2011) (Fig. 1).

2.7 Pandemic/2009-like H1N1 and its Variants

From the time of the peak of the human pandemic, the pandemic H1N1/09-like virus has been repeatedly isolated from pigs in many Asian countries (Vijaykrishna et al. 2010; Song et al. 2010). Most of these pdm/09-like swine isolates resulted from different direct introductions from humans to pigs. However, the detection of multiple reassortant viruses between pdm/09 and other swine viruses (Vijaykrishna et al. 2010; Starick et al. 2011; Moreno et al 2010), along with the high seroconversion rate to the pandemic virus in pigs (unpublished data), suggest that this virus might gradually become established in this host. Questions remaining to be answered are when that will occur, what kind of genetic composition the established virus will have and what its long-term impact will be.

2.8 Avian-like Influenza Viruses

In the last two decades avian influenza viruses have frequently been isolated or detected from pigs in Asian countries, likely due to increased farming activity and interaction between pigs and birds. However, none of these interspecies transmission events caused severe consequences or the establishment of avian origin viruses or virus genes in pigs. The most frequently detected avian viruses in pigs in Asian countries belong to the virus lineages that are long-term enzootic in poultry, such as H9N2 and H5N1 viruses. H9N2 avian-like viruses were detected in China and Korea, and H5N1 avian-like viruses were reported from China, Vietnam and Indonesia (Nguyen et al. 2005; Nidom et al. 2010; Yu et al. 2011). All H9N2 and H5N1 swine virus isolates were from different sublineages and variants, highlighting the long-term potential threat from these viruses.

Other subtypes of avian influenza viruses detected in pigs in Asian countries include H1N1, H3N2, H5N2, H11N6 and H6N6 (Guan et al. 1996; Zhang et al. 2011; Lee et al. 2009; Kim et al. 2010; Kida et al. 1988). Genetic analyses revealed that these viruses were likely derived from those residential in aquatic birds. Almost all were detected only on a single sampling occasion, but some were from disease surveillance in pigs and low seroconversion rates were observed (Zhang et al. 2011; Lee et al. 2009). This shows that pigs are susceptible to most subtypes of avian influenza viruses. A major concern is that should highly pathogenic H5N1 Asian lineage viruses recruit mammalian-adapted virus genes from pigs, a human-to-human transmissible virus might be generated. Systematic surveillance in different countries would be greatly helpful to counteract such an event.

3 Summary

The findings presented here suggest that almost all major swine influenza virus lineages from different continents are co-circulating in pigs in Asian countries. The movement of live pigs between different continents is probably responsible for this. Co-circulation of these different virus lineages will naturally increase virus interaction and reassortment, and the genetic diversity in swine influenza viruses. The emergence of the 2009 pandemic H1N1 virus provided the clearest example of the potential consequences of co-circulating viruses even though we lack evidence to show that this pandemic virus was initially generated in Asian countries. Given that all current swine virus lineages and their constituent segments have been prevalent for more than a decade in pigs (i.e. they are fully mammalian-adapted) and H9N2 and H5N1 avian influenza viruses are widely enzootic in poultry in the region, generation of a novel virus with efficient transmissibility in pigs or even in humans is possible.

Acknowledgments This work was supported by the National Institutes of Health (National Institute of Allergy and Infectious Diseases contract HSN266200700005C); Li Ka Shing Foundation; and Area of Excellence Scheme of the University Grants Committee of the Hong Kong SAR (grant AoE/M-12/06).

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Clinicopathological Features of Swine Influenza

B. H. Janke

Abstract In this chapter, the clinical presentations, the development of infection and the macroscopic and microscopic lesions of swine influenza virus (SIV) infection are described. Both natural and experimental infections are discussed.

Contents

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Current Topics in Microbiology and Immunology (2013) 370: 69–83
DOI: 10.1007/82_2013_308

Published Online: 13 February 2013

1 Introduction

As the worst global human pandemic climaxed during the summer of 1918, a new disease entity began to be recognized in swine in the Midwestern United States. The clinical signs of this new disease readily differentiated it from classical swine fever (hog cholera), which was the infectious disease of most consequence for swine at that time. This new respiratory disease was tabbed "hog flu" because of the similarity of the disease to influenza in humans (Dorset et al. 1922).

An early description paints a memorable clinical picture: "The onset of hog flu, as already stated, is sudden, an entire herd coming down, as a rule, within a day or two…The first symptom noted is loss of appetite, the animals failing to come up for their feed. They are disinclined to move and lie around the straw stacks or in their houses. When temperatures are taken, the animals are found to have fever. A thumpy or jerky respiration soon develops which is best observed when the animals are lying down and at first may be so slight as to escape notice unless the animals are carefully watched; later, it becomes more pronounced and may be noted when animals are standing. The disease evidently has a very short incubation period and develops rapidly. The second or third day, the entire herd, as a rule, will be lying in their nests and often present a very sick appearance. Sometimes one may walk among the sick animals and even step over them without rousing them, and anyone viewing for the first time a herd suffering from hog flu at the height of the infection would probably think that most of the affected animals would succumb. When the sick animals are roused from their nests, they almost invariably cough. The cough is paroxysmal in character, the back being often arched, and the spells of coughing are sometimes of sufficient violence to induce vomiting; in this respect the disease resembles whooping-cough in the human. When the paroxysms of coughing have passed, the animals stand in a listless attitude with their heads down, their tails limp, and soon lie down as though tired. The sick animals usually rest on their bellies, and sometimes assume a partly sitting position with the body propped on the forelegs, as if to afford room for greater lung expansion. There is usually a conjunctivitis, characterized by a watery or gummy secretion from the eyes, and a nasal discharge may also be present……" (McBryde 1927).

2 Clinical Disease

2.1 Classical Epidemic Swine Influenza

Epidemic swine influenza as described above was the predominant presentation of the disease in the United States for nearly 70 years. Indeed, this classical virus and presentation of swine influenza in conventional swine populations continues today as an acute, high morbidity-low mortality infection that spreads rapidly through groups of pigs. For the first 1–2 days after infection, affected pigs develop high

fevers (>105°F, 40.5 °C) with lethargy and anorexia. Close examination reveals clear nasal discharge and conjunctivitis. Tachypnea and expiratory dyspnea (thumping) are often pronounced, especially when pigs are forced to move. By days 3 and 4, pigs begin to acquire the hallmark clinical sign of this disease, a harsh deep barking cough that results from the extensive bronchitis and bronchiolitis. In many pigs, fevers will have begun to drop by this time. Pigs of all ages, from mature sows to nursery pigs, may be similarly affected, but clinical disease is often milder in nursing pigs. In some outbreaks, sows may be inappetent and lethargic and develop high fevers but have less prominent respiratory clinical signs. Pregnant animals may abort. In the absence of concurrent bacterial pneumonia, individual pigs recover quickly, usually within 6–7 days. Not all pigs in a group will be infected simultaneously, and the disease course for the entire group may require a 2+ weeks before clinical signs abate and pigs return to normal body condition and weight gain. Mortality is generally low although some virus strains exact a higher toll.

Historically, there has always been a distinct seasonality to swine influenza in the north central U. S., a part of the country that has widely divergent seasonal temperatures. In years past, outbreaks commonly occurred each fall, with little recognition of the disease at other times of year. Even under current confinement production systems, the disease still exhibits a consistent seasonality, with the greatest peak in the fall and a smaller peak in the spring, the seasons of transition for prevailing weather conditions (Janke et al. 2000). Wide swings in temperature over short periods of time make it difficult to modulate housing environments, and these climatic stresses may increase animal susceptibility to infection. Cool moist conditions contribute to environmental survival and aerosol spread of the virus. How and where the virus survived/survives between outbreaks has never been fully explained. A long-term carrier state has not been discovered, and most individual pigs appear to clear the virus within 2 weeks. The virus is likely maintained in herds by subclinical passage to naïve pigs or those with low or compromised immunity. Studies on vaccine efficacy have indicated that there is no absolute immunity threshold for swine influenza virus (SIV) infection. Pigs with sufficient immunity to prevent clinical illness can still be infected and shed virus, though it is much reduced in duration and titer (Richt et al. 2006; Van Reeth et al. 2001).

2.2 *Current Clinical Expression in Larger Swine Populations in Segregated Rearing Production Systems*

Many pigs today are raised in segregated rearing systems. Sows are maintained, bred, and farrowed at a location separate from the farms on which younger pigs are fed to market weight. Once farrowed, pigs are only kept with the sows for about 3 weeks before they are weaned and moved to nurseries on another site (segregated early weaning). In two-site systems, pigs will be raised at that location until being sent to market. In three-site systems, pigs will be fed at the nursery site until

about 10–12 weeks of age, after which they are moved to grow-finish units at a third location. At each location, the goal is to fill and empty each building completely at one time (all-in all-out). The objective is disease control, i.e., to minimize situations in which infections can be passed from older immune pigs on a site to younger naïve pigs as they are brought into the same buildings. In continuous flow systems, viruses and bacteria have greater opportunity to maintain contagious levels because of the periodic addition of susceptible hosts. This age-based segregation and movement of pigs must be considered when considering the clinical presentation of swine influenza as it currently manifests itself. In nearly all swine production units, one can find influenza virus in circulation or serologic evidence of previous exposure.

At the present time, because of the almost universal immunity against SIV at some level against some variant in all herds, and the number of subtypes, antigenic clusters within subtypes and reassortants that circulate within swine populations, expression varies widely. Outbreaks still occur but infections are more frequently endemic with clinical expression more muted and melded with that of other concurrent respiratory infections. Pigs of all ages from nursing pigs to sows may be infected, with blips of clinical disease appearing at different phases of production that vary with the situation. It becomes the task of veterinary practitioners and diagnosticians to determine whether increased clinical disease is due to the resurgence of a virus already circulating within the operation or to the introduction of a different virus against which only partial immunity may exist.

Expression of disease generally falls into one of the following presentations:

1. Acute, “fulminating” SIV—Resembling disease as it was first observed 90 years ago but now the least frequent presentation, this is the outbreak of a pathogenic variant in a population that has little or no specific antibody to attenuate the infection. There is very rapid spread, high fevers, anorexia, expiratory dyspnea with effort, mortality, and a brief course in the herd/group. Mortality occurs in pigs whose lungs fill rapidly with fluid; a typical ‘foam cast’ forms in the bronchial tree and is expelled through the nose. There is very rapid spread within and between sites and through pigs of all ages. Even nursing pigs are severely affected, but essentially all survive. Recovery is rapid and often complete, with very few secondarily infected or chronically affected pigs, and infection is followed by very high levels of specific immunity. This is rarely confounded with other agents even though they may be present.
2. Age-associated influenza in growing pigs—Infection can be relatively predictable in certain systems depending on how they are structured. Maternal passively acquired antibody is protective for weaned pigs against endemic virus, but that immunity wanes with maternal antibody decay. Usually occurring when sow herd immunity is uniform, and pigs have roughly equivalent levels of passive protection, infection that is clinically evident is delayed until the pigs are exposed to homologous or variant virus that can ‘break through’ the collective passive immunity. The classic pattern is robust pigs with no problems through the nursery phase until they are 10–12 weeks old and in finishers where

they are exposed to other older growing pigs that are shedding virus. Approximately 2 weeks into the finishing phase, pigs develop cough and depression that does not explode, but rather works its way through the group over a protracted 2–3 week timeline as the individual passive antibody decay curves meet up with the various loads of virus that overcome it. The actual SIV-induced clinical disease expressed is usually not dramatic, of variable severity and commonly complicated by concurrent infections endemic in the group.

3. Piglet influenza—A consistent/persistent clinical expression of SIV can be found in nursing to newly weaned pigs. Again associated with passive protection variance within groups and increasing frequency of SIV variants, clinically the infection is first appreciated as a cough in the 2–3-week-old pig about to be weaned. But the cough is in scattered pigs in a given room (maybe 10 % of litters have a pig that sporadically coughs) that are hard to find and almost too subtle to raise any alarm. When these pigs are moved to the nursery, the stress and activity exacerbate clinical expression. During the move and on first entry into the nursery, there is a common description "…newly weaned pigs came off the truck coughing…" even though the farrowing management would say "…no, we didn't notice any cough; maybe a pig or two…". Clinically in this situation, a subset of pigs suffers fever, anorexia, and after a couple days, cough due to SIV infection. Again, the severity of clinical disease is greatly dependent on the level and specificity of passive protection. Affected pigs are anorexic during the critical transition phase to solid food, i.e., they are sick for 36 to 48 h after the move, and become very hard to start on feed. When just a few pigs in a large group are affected, the infection probably goes unrecognized, but on many farms, a consistent 5–10 % or greater are affected and the problem is economically substantial. These situations are the purpose of sow immunization, not to protect the sow, but to try to extend the passive shield until pigs are well-started on feed and can handle the infection.

2.3 Clinical Disease in Experimentally Infected Pigs

The clinical disease induced by experimental challenge rarely reaches the severity of that observed in the field (Landolt et al. 2003; De Vleeschauwer et al. 2009). Clinical signs of illness (fever, depression, anorexia, tachypnea, serous nasal, and ocular discharge) develop to some degree after most challenges but vary under the multitude of protocols employed (Nayak et al. 1965; Winkler and Cheville 1986; Brown et al. 1993; Van Reeth et al. 1996; Thacker et al. 2001; Richt et al. 2003; Landolt et al. 2003; Jung et al. 2005; Vincent et al. 2006; De Vleeschauwer et al. 2009; Sreta et al. 2009). Under most experimental models, tachypnea is evident in pigs at least when aroused, but coughing is usually minimal, limited to an occasional soft cough that develops a day or two after the onset of other clinical signs. Fever is quite variable as is the onset of clinical signs. In general, with higher virus

titers in inoculum (>10^6–10^7 $TCID_{50}$, EID_{50} or PFU/pig) and intratracheal inoculation, the onset of illness occurs sooner [24–36 h postinoculation (PI)] and clinical signs are more apparent. With lesser challenge regimens (10^3–10^5 $TCID_{50}$, EID_{50}, PFU/pig) and intranasal inoculation, clinical disease may not be seen or may take 2–4 days to become evident. Duration of clinical illness is usually 2–4 days with most experimental infections.

3 Virus Infection, Replication, and Shedding

SIVs are spread between pigs through direct contact via nasal secretions and through inhalation of aerosolized virus in droplets generated by coughing and exhalation. The cellular targets of infection are the epithelial cells lining the nasal passages, trachea, bronchi, bronchioles, and alveoli. Numerous experimental infection studies have been conducted to define the progression of the infectious process.

A variety of inoculation methods have been employed in these experimental infection studies to administer virus: nebulization via nose cone or chamber, direct intranasal (IN) inoculation, and intratracheal (IT) inoculation. Nebulization is probably the most effective method of depositing large quantities of virus throughout the respiratory tract but this method is also the most labor intensive and is little used. Intranasal methods (the mechanical specifics of which are often not described) appear to be more variable in their efficacy of establishing infection, probably because with some techniques pigs swallow most of the challenge dose. Intratracheal methods provide the most consistent challenges, and if not deposited too far down the tract, the virus appears to be well-distributed throughout the lungs.

Infection and multiplication in host cells progresses very rapidly with influenza viruses. In an immunofluorescent study (Nayak et al. 1965), the first evidence of virus infection was a pale fluorescence in the nucleus of bronchial epithelial cells, as early as 2 h PI. By 4 h PI, virus antigen was abundant in both nucleus and cytoplasm of infected cells. In an ultrastructural study (Winkler and Cheville 1986), virus was observed budding from the surface of Type II pneumocytes as early as 5 h PI.

Only low numbers of randomly scattered cells are observed in the nasal turbinates and trachea during the first 24–72 h PI (Nayak et al. 1965). The most extensive infection occurs in epithelial cells lining the bronchi and bronchioles, with peak infection occurring at 48–72 h PI. Although some virus reaches the alveolar level early, especially with nebulization or high dosages with other methods, more extensive spread of virus to alveolar epithelial cells tends to occurs later in the course of infection, at 72–96 h PI (Jung et al. 2005; Van Reeth and Pensaert 1994).

Multiple studies (Brown et al. 1993; Van Reeth et al. 1996; Landolt et al. 2003; Vincent et al. 2006, 2009a, b; De Vleeschauwer et al. 2009; Ma et al. 2010) report nasal virus shedding by 1–3 days PI, regardless of the route of inoculation, and the duration of shedding, for 4–5 days, occasionally to 7 days PI. In one study, intranasal inoculation (10^7 EID_{50}) resulted in virus shedding by 24 h PI, whereas intratracheal inoculation resulted in nasal shedding being delayed until 72 h PI and the peak titer of virus shed was much lower (De Vleeschauwer et al. 2009). In another study using intranasal inoculation, lower virus titers (10^3–10^4 $TCID_{50}$) resulted in 24 h delay in onset and peak of nasal shedding compared to pigs given 10^5–10^6 $TCID_{50}$ although peak titers of shed virus were similar (10^7 $TCID_{50}$/ml) (Landolt et al. 2003). The amount of virus shed in nasal secretions tended to be fairly consistent through days 2–4 PI with peak titers described in the range of $10^{3.5}$–$10^{7.5}$ $TCID_{50}$, EID_{50}, or PFU/ml of fluid used to flush nasal passages or to wash virus from nasal swabs.

Determination of virus titers in lung homogenate or bronchoalveolar lavage fluids is often used in experimental studies to monitor the dynamics of virus production in the lung. Peak virus load in the lung, as measured in studies that cover the first few days of infection, occurs at about day 3 PI with titers varying from $10^{4.5}$ to $10^{8.3}$ $TCID_{50}$, EID_{50} or PFU/ml. Titers hold at relatively similar levels through 5 days PI. (Van Reeth et al. 1996; De Vleeschauwer et al. 2009; Vincent et al. 2009a, b; Ma et al. 2010).

The narrow time frame of virus replication and shedding described above is consistent in most challenge trials for most influenza viruses isolated from swine regardless of subtype. Experimentally, the dose of virus that reaches the lung initially may affect the course and severity of experimental infection. If low doses are given, either intranasally or intratracheally, the virus may initially spread more slowly which may result in a delayed onset and ultimately milder course of clinical disease. With most viruses, the course of infection is short and essentially complete within 5–7 days. The comparative effects of virus titer in inoculum, route of inoculation and/or age on the dynamics of infection have been described in several studies (Landolt et al. 2003; Richt et al. 2003; De Vleeschauwer et al. 2009).

In swine, influenza virus infection is generally considered to be limited to the respiratory tract, but a few studies have reported virus in extra-respiratory sites. A few infected cells were detected by immunohistochemistry in mediastinal lymph nodes, but none were detected in tonsil (Nayak et al. 1965). Influenza virus was isolated from the serum of all five inoculated pigs, for only one day each, at 1–3 days PI (Brown et al. 1993). In a more recent study, virus was detected by RT-PCR in spleen, ileum, and colon but not by virus isolation. Virus was detected in brainstem by both PCR assay and virus isolation, but no specific infected cells were detected by IHC (De Vleeschauwer et al. 2009). In this paper, researchers referred to unpublished data from in vitro studies indicating this virus could infect porcine trigeminal ganglion via the axons.

4 Pathology

4.1 Macroscopic Lesions

The most common macroscopic manifestation of influenza virus infection is a cranioventral bronchopneumonia that can affect a variable amount of the lung. The lesions are similar in both experimental inoculation and uncomplicated natural infections (Janke 1998) (Fig. 1a, b). Such expression would be expected since the virus enters the lung via the airways rather than through viremia. In milder infections, dark red multilobular to coalescing, often somewhat linear, foci of consolidation are evident in the hilar area and more dorsal portions of the cranial and middle lung lobes. More extensive infections involve larger, usually more ventral, portions of the cranial and middle lobes and cranioventral portions of the caudal lobe; as much as 40 % of the total lung volume may be affected. In field cases, the lesions often involve concurrent bacterial bronchopneumonia which results in more extensive lesions. In an occasional pig, a few hemorrhagic emphysematous bullae distending interlobular spaces may be evident. Tracheobronchial lymph nodes are variably swollen and congested. The trachea and nasal turbinates may be congested but are usually unremarkable. Although virus infects the epithelial lining of these upper airways, grossly visible necrosis does not develop.

Less frequently encountered in field situations, and not reproduced by experimental challenge, severe acute influenza infections may result in a diffusely congested and edematous lung with abundant foam in the trachea and larger airways (Janke 1998). In such an acutely affected lung, cranioventral lobular consolidation may be obscured by the diffuse inflammation.

In experimental studies (Winkler and Cheville 1986; Van Reeth et al. 1996; Thacker et al. 2001; Richt et al. 2003; Landolt et al. 2003; Vincent et al. 2006, 2009a, b; De Vleeschauwer et al. 2009; Sreta et al. 2009; Ma et al. 2010) the extent of lung involvement also is quite variable and usually is expressed as percent of total lung affected, calculated either by addition of the portions of each lobe (Halbur et al. 1995) or as the average for all lobes, with described values ranging from <1 to 58 %. As with clinical signs and virus distribution and shedding, lesion severity is influenced by the route of inoculation and the virus titer in the inoculum. An inoculum containing 10^6 or higher infectious doses of virus introduced intratracheally will often result in 10–30 % lung involvement. Inocula with 10^3–10^4 $TCID_{50}$ or EID_{50} of virus, especially if administered intranasally, may result in <10 % lung involvement. In some experimental infections, the dorsocaudal aspect of the caudal lobe also is affected, most likely an artifact of the method of inoculation as this presentation is unusual in field cases.

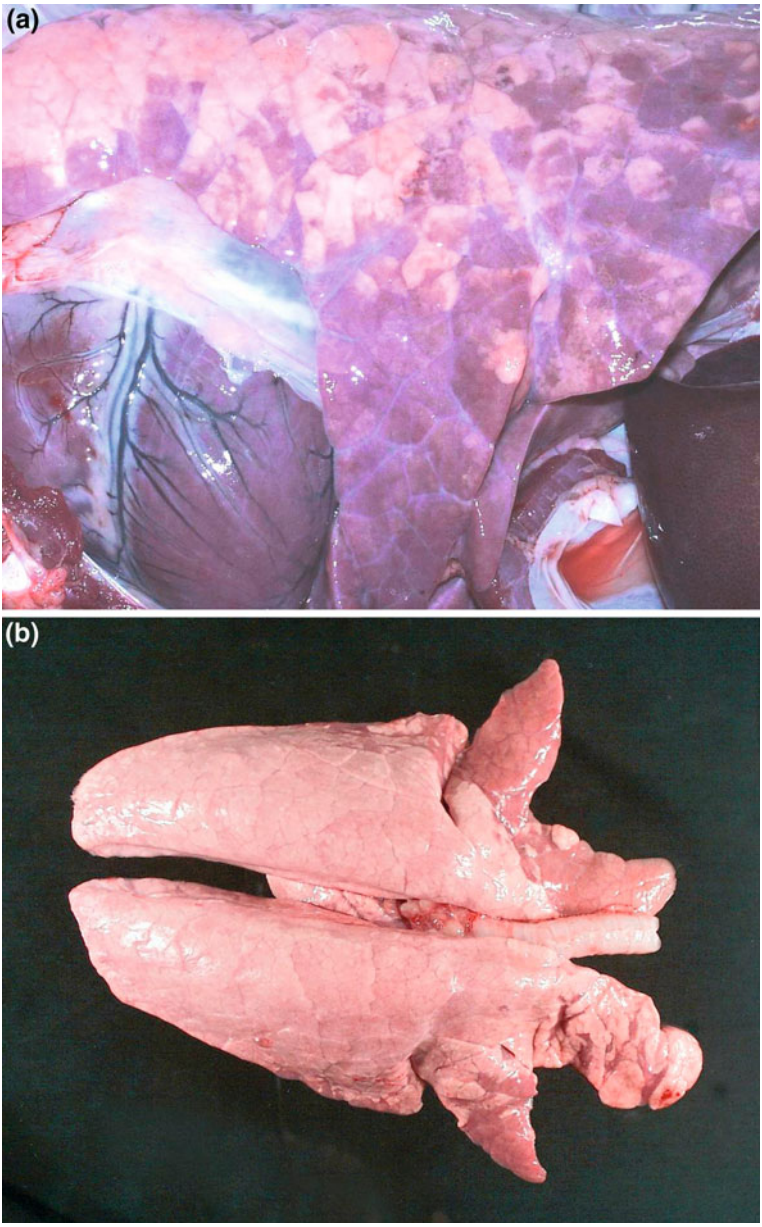

Fig. 1 **a** Swine influenza in a grow-finish pig (field case). Lobular and sublobular consolidation affecting a large portion of cranioventral lung. **b** Lungs from a 6-week-old pig experimentally inoculated with H3N2 SIV and euthanized 5 days postinoculation. Multifocal to coalescing consolidation in cranioventral portions of lung

4.2 Microscopic Lesions

The two most detailed microscopic descriptions of the effects of SIV infection on swine respiratory tract are a histopathologic and immunofluorescent study (Nayak et al. 1965) and an electron microscopic (ultrastructural) study (Winkler and Cheville 1986), both of which are experimental challenge studies with classic H1N1 virus. Less comprehensive but similar descriptions of microscopic lesions induced by SIV infection, some with concurrent immunohistochemical (IHC) studies describing virus distribution in tissues, have been reported by numerous researchers in studies characterizing isolates of interest (Brown et al. 1993; Van Reeth et al. 1996; Landolt et al. 2003; Jung et al. 2005; De Vleeschauwer et al. 2009; Sreta et al. 2009). Additional descriptions of the effect of SIV infection in pigs can also be found in many other studies, often in comparison to human and avian viruses inoculated into swine or in vaccine trials. The descriptions below are composites drawn from these studies as well as the author's experience with both field cases (Janke 1998) and experimental trials (Thacker et al. 2001; Richt et al. 2003, 2006; Solorzano et al. 2005; Vincent et al. 2006, 2007, 2008, 2009a, b; Kitikoon et al. 2009; Ma et al. 2010). The hallmark microscopic lesion of influenza infection, consistently present, is necrotizing bronchitis and bronchiolitis (Fig. 2a). Interstitial pneumonia, though usually evident to some degree, is quite variable in severity in both field cases and in experimental trials, often with pig-to-pig variation.

The earliest response to infection is neutrophil infiltration. By 4–8 h PI, neutrophils are emigrating through airway epithelial layers and accumulating in the lumens of alveolar capillaries. Endothelial cells lining the capillaries are swollen and pavementing of vessel walls by marginated neutrophils is evident. Alveolar walls are widened by vascular congestion and lymphatic dilation. Although ballooning degeneration and cytoplasmic vacuolization of some epithelial cells lining smaller bronchioles may be recognized as early as 8–16 h, these changes are subtle and scattered, and most airways are still intact.

By 24 h PI, extensive infection of epithelial cells lining scattered airways of variable size can be detected by IHC. In a few studies, airway epithelial necrosis was described at this time, but in most studies, disruption of the epithelial layer in a significant number of airways has not yet occurred. Small numbers of neutrophils may be clustered in some airway lumens, accompanied by light infiltration of lymphocytes around some bronchioles. Alveolar walls may be more prominently thickened by congestion, edema, and leukocyte infiltration, predominantly peribronchiolar in distribution.

By 48 h, there is extensive necrosis and sloughing of epithelial cells into airway lumens accompanied by more obvious neutrophil accumulation. Loose infiltration of lymphocytes around affected airways is more prominent but still light. The epithelial cells remaining attached are swollen or attenuated and the layer is irregular in outline. Thickening of alveolar walls, if prominent, is more diffuse. Pneumocytes lining alveoli may be swollen with some sloughing into the lumen.

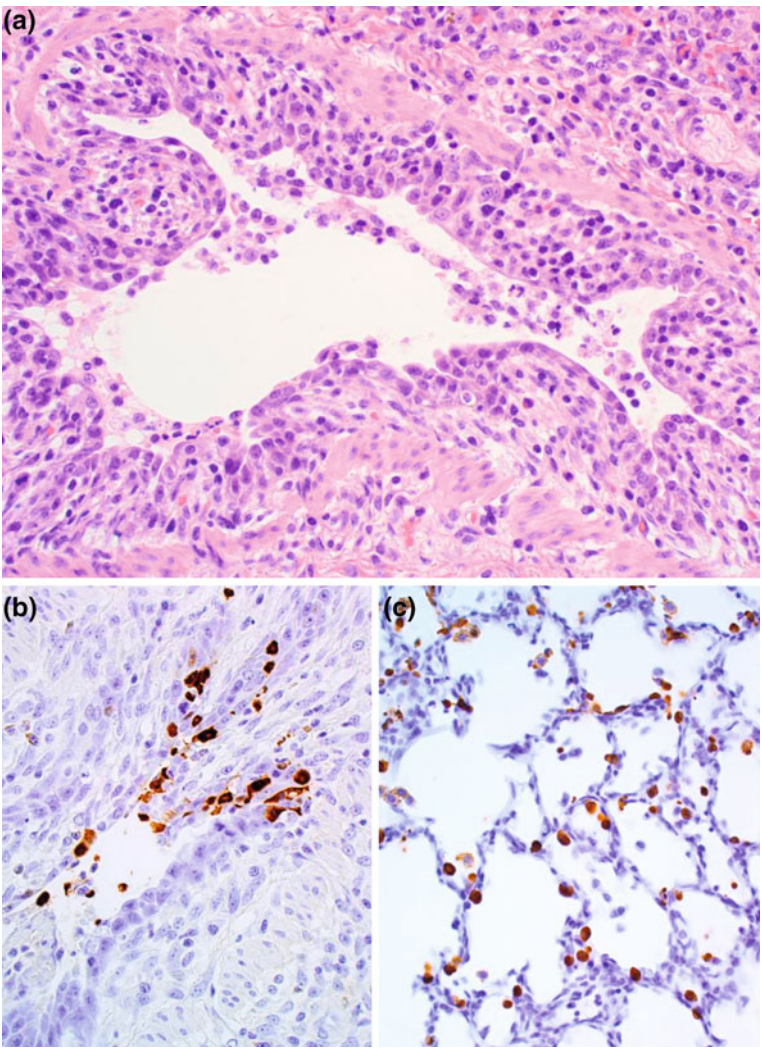

Fig. 2 **a** Subacute necrotizing bronchiolitis in the lung of a 6-week-old pig inoculated intratracheally with H3N2 SIV and euthanized at 3 days postinoculation. Extensive necrosis and sloughing of epithelial cells from a segmental bronchiole is evident. HE x40. **b** Immunohistochemical staining of a similar bronchiole from the same pig identifying virus-infected epithelial cells sloughing into the lumen. IHC x40. **c** Immunohistochemical staining of alveoli from the lung of a 6-week-old pig inoculated with H1N1 SIV by nebulization and euthanized at 24 h postinoculation. Virus has penetrated deep into the lung and numerous pneumocytes lining alveolar walls are infected. IHC x40

Numerous epithelial cells in affected airways (Fig. 2b), both attached and sloughed, contain virus antigen by IHC, but only a few individual to small clusters of infected cells will be observed in alveoli. Some of these cells are obviously

swollen pneumocytes still attached to alveolar walls or sloughed into the lumen (Fig. 2c), but other cells within the alveolar wall or loose in the alveolar lumen appear to be macrophages. By close examination, necrotic constituent cells may be identified in alveolar walls, but the septa remain intact. In occasional severe infections, clumps of necrotic cell debris are evident in clusters of alveoli. Consistent with the macroscopic appearance, lobules within the same section of lung may differ in the degree of involvement. Severely affected lobules are frequently atelectatic.

In both field cases and experimental challenge studies, the sizes of airways that are affected may vary. Most likely due to the dynamics of airflow that affect droplet suspension and deposition or to receptor distribution, the largest airways without cartilage (primary bronchioles) are most consistently and most severely affected (Thacker et al. 2001). In some animals, both experimentally and in field cases, the larger lobar or segmental bronchi may be spared. Conversely, in some mild infections, only these larger airways may be infected. The smallest terminal or respiratory bronchioles may be spared or may be necrotic, and the degree of alveolar involvement varies. Except in severe cases, the lesions are multifocal and unaffected lobules sit adjacent to severely affected lobules.

By 72 h PI, some airways are in active necrosis and filled with debris, but many airways are lined by an intact hyperplastic epithelial layer. Peribronchiolar lymphocytic cuffs are well-developed. Alveolar walls may still be thickened as described above with a light loose mixed population of sloughed pneumocytes, macrophages and leukocytes residing in alveolar lumens. Leukocyte populations have shifted to predominantly mononuclear cells. By this time, few infected airway epithelial cells will be identified by IHC but more numerous scattered infected cells, often limited to certain lobules, will be detected in alveoli. In some infections, alveoli may be little affected.

By 96 h and beyond, airways are in repair, lined by hyperplastic or nearly normal epithelium and surrounded by moderate-sized lymphocytic cuffs. Alveolar inflammation is also resolving. By this time, very little virus can usually be detected by IHC, in occasional isolated airways or in scattered individual cells in alveoli. In field cases, in some severely damaged bronchioles, repair is accompanied by fibrosis and endobronchial polyp formation (bronchiolitis obliterans). Such lesions are rarely observed in experimental infections. Over the following days, the epithelial hyperplasia resolves and peribronchiolar lymphocytic cuffing and partially atelectatic alveoli with variable leukocyte populations are all that remain. Though somewhat dependent on the extent of damage, lungs return to normal by 2 weeks PI.

In trachea, infected epithelial cells, as identified by IHC, are usually few in number and widely scattered. Damage to the tracheal epithelial lining, as characterized by attenuation or squamous metaplasia, if present at all, tends to be focal to multifocal. Very few viruses induce extensive epithelial injury, and even then, not consistently in all pigs. Subepithelial lymphocyte infiltration may be intense in the latter situations, but in most tracheas with minimal or focal epithelial attenuation, inflammation exhibits the same range of variation in severity as that

observed in pigs not challenged with SIV. Although pigs shed quantities of virus over multiple days in nasal secretions, only mild attenuation of the epithelium lining the inside of nasal turbinate scrolls is observed and that inconsistently. Only low numbers of infected cells are usually identifiable in histopathologic sections by IHC. Infection of tonsil and tracheobronchial lymph node has been reported in some studies described above. In the author's experience, low numbers of infected cells can be identified by IHC in the lymph nodes but rarely in tonsil.

This rapid sequence of events will occur in both the individually affected lobules in most naturally infected pigs and in pigs experimentally infected with high doses of virus. Under experimental challenge conditions in which less virus may be given, examination of multiple sections of lung may reveal asynchronous infection with different lobules at different stages of infection.

5 The Question of Virulence

Although some outbreaks of epidemic swine influenza suggest that certain viruses may be more pathogenic or virulent than others, defining or discovering the basis for virulence has proven to be difficult. Even viruses from severe field outbreaks have tended to be rather tame in captivity (Ma et al. 2010). Most comparative studies conducted in tandem under the same protocols have revealed only minor differences between viruses. More virulent viruses have been deemed so, not so much on increased severity of clinical disease, but on higher or more prolonged fever, higher or prolonged virus shedding or titers in lung, and more extensive macroscopic lesions. Microscopically, lesions tend to be similar but with more lobules and more airways within lobules affected.

Comparative evaluation of results of studies from different researchers must be done cautiously because of the multitude of factors (virus titer, route, and method of inoculation, age of pigs, etc.) that can affect results. Thus, the most significant observations in this regard are likely to come from researchers who have used similar protocols in multiple animal trials with many different viruses. Clinical differences are usually subtle but analysis of parameters that can be quantified can yield clues to virulence differences, e.g. fever, cytokine levels, virus shedding, virus titers in lung tissue or BALF, gross and microscopic lesion scoring. (See Landolt et al. 2003; Richt et al. 2003 for examples of scoring). Although differences may be minimal or below statistical significance because of the number of animals that researchers can reasonably afford to use in such trials, the various parameters often correlate well. In the field situation, such differences would likely be amplified. Some studies suggest the newer triple reassortant viruses may be more virulent than preceding classic viruses (Vincent et al. 2006; Ma et al. 2010). Several recent triple reassortant viruses recovered from situations in which both pigs and people were infected also appear to be slightly more virulent in swine from which they originated (Vincent et al. 2009b).

Most of the research into virulence factors in influenza viruses has been initiated with two unquestionably virulent viruses: H5N1 highly pathogenic avian influenza virus and reconstructed 1918 human pandemic virus (Tumpey et al. 2005; de Wit and Fouchier 2008; de Wit et al. 2008; Basler and Aguilar 2008; Lycett et al. 2009; Janke et al. 2010). The reverse genetics techniques now available allow researchers to replace specific genes or gene sequences in influenza viruses to compare the relative contribution of each gene to virulence or infectivity. Pigs are one of the models (mice, ferrets, chickens, primates) used for such studies, and information gained from such studies may eventually benefit understanding and control of SIV infection (Memoli et al. 2009; Solorzano et al. 2005; Richt et al. 2006).

Acknowledgments The author would like to gratefully thank Dr. Steve Henry, Abilene Animal Hospital, Abilene, KS for his contribution on the current presentation of swine influenza in the field and Dr. Kent Schwartz, Iowa State University, Ames, IA for his contribution of knowledge and insight into all things pig.

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Diagnostics and Surveillance for Swine Influenza

Susan Detmer, Marie Gramer, Sagar Goyal, Montserrat Torremorell and Jerry Torrison

Abstract Collective knowledge regarding the occurrence of influenza among swine is incomplete due to inconsistent surveillance of swine populations. In this chapter, we review what surveillance activities exist and some of the practical challenges encountered. Furthermore, to support robust surveillance activities, accurate laboratory assays are needed for the detection of the virus and viral nucleic acids within clinical samples, or for antiviral antibodies in serum samples. The most common influenza diagnostic assays used for swine are explained and their use as surveillance tools evaluated.

Contents

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Current Topics in Microbiology and Immunology (2013) 370: 85–112
DOI: 10.1007/82_2012_220

Published Online: 8 May 2012

1 Surveillance for Influenza Viruses in Swine

Influenza A viruses in swine typically cause an acute respiratory disease which, in uncomplicated cases, is mild and self-limiting (Radostits et al. 2000). Infection of swine with influenza A virus is common (Brown 2000) and occurs throughout the year (Vincent et al. 2008). However, seasonal peaks occur in months with moderate temperatures and humidity (Shaman and Kohn 2009) similar to the pattern of disease seen in humans. Because endemic swine influenza is highly prevalent but causes minimal mortality in infected pigs, the World Organization for Animal Health (Office International de Epizooties), and the U.S. Department of Agriculture (USDA) have not classified swine influenza as a notifiable or reportable disease (OIE 2009; USDA/APHIS 2009). Further complicating the coordinated surveillance efforts are the limited resources available for animal disease surveillance in general. For these financial and biological reasons, systematic and rigorous surveillance is focused on diseases of much higher consequence to animal health and international trade, such as brucellosis and foot-and-mouth disease. Animal disease surveillance in general is labor-intensive and costly and hence animal health authorities at the international, national, provincial, and state levels have precluded assigning it a higher priority for funding (Pappaioanou and Gramer 2010). Given these challenges, the efficient and effective surveillance of influenza viruses in swine will require a strategic approach, encompassing all the attributes of a successful surveillance program.

1.1 Attributes of Disease Surveillance Systems

When considering an influenza virus surveillance program for swine populations the key attributes of disease surveillance systems developed and used by leading public health authorities for detecting diseases of public health importance in human populations (CDC 2009) must be considered. These attributes are summarized below.

A. *Simplicity*. This refers to the surveillance system's structure and ease of operation. As with most successful operations, systems that prove to be the most valuable utilize methods that are as simple as possible while still fulfilling the primary objectives.
B. *Flexibility*. A system that can adapt to changing needs, such as the addition of new collection methods or employing new and more specific diagnostic assays has built-in flexibility to capture the required information.
C. *Acceptability*. A surveillance system must appeal to all interested parties and, once found acceptable, it reflects the willingness of individuals and organizations to participate in the surveillance system (e.g., swine farmers, veterinarians, and veterinary diagnostic laboratory personnel who are asked to report cases of disease).

D. *Timeliness*. After initial diagnosis, how quickly the cases are entered into the surveillance system or the time that elapses between onset of infection, diagnosis, case report, information sharing, and action, is often regarded as key to a surveillance system's success (Jajosky and Groseclose 2004). While timeliness is of critical importance, it is often very difficult to measure (Jajosky and Groseclose 2004).
E. *Completeness*. Completeness is the attribute of a surveillance system that is most directly linked to the true discipline of epidemiology. Completeness is reflected by the proportion of all cases of disease in a specified population that are detected by the surveillance system and is affected by the likelihood that: (a) animals with infection or disease are tested; (b) the condition is correctly diagnosed (skill of animal health provider, accuracy of diagnostic tests); and (c) the case is reported to the surveillance system once it has been diagnosed. The factors that may affect completeness of a surveillance system are addressed in more detail in Sect. 1.3.
F. *Representativeness*. A surveillance system that accurately describes the occurrence of disease over time and its distribution in the study population by location, group, and severity can be referred to as representative. Consideration of this attribute is especially important for large populations with variable prevalence because most systems simply cannot detect every single case of infection or disease. The common idiom "tip of the iceberg" is a popular way of referring to how the documented and described cases of a disease that are evident as the result of a surveillance program truly represent the largely undetected/unseen cases in the vast population.

While there are several challenges that inherently exist when trying to conduct surveillance on animal populations (further discussed in Sect. 1.3), if an organization is forward thinking and keeps these key attributes in mind, a wealth of information can be generated. The information garnered from an influenza surveillance system for pigs is driven in large part by the initial design and rationale for the surveillance.

1.2 Rationale Behind Influenza Surveillance Systems for Swine

While the majority of a surveillance effort is designed to answer "how" and "what", e.g., logistics and population selection, the better part of the planning time should be devoted to determining "why" to invest in the activity in the first place. For influenza virus surveillance in animals, the simple, altruistic reason is that surveillance must be done to protect public health and prevent pandemics (Patriarca and Cox 1997). Philanthropic intentions aside, it is important to find rationale for surveillance that will also benefit or provide information to all stakeholders. A recent Institute of Medicine (IOM) review for the National Academies, "Sustaining Global Surveillance and Response to Emerging Zoonotic

Diseases," hits the nail on the head with its recommendations to improve early detection and response to zoonotic diseases, such as influenza. Specifically, they are of the opinion that comprehensive surveillance would be best achieved in the following manner: "Multidisciplinary teams of professionals that have relevant expertise and field experience would identify populations at risk and causes and risk factors for infection, and then rapidly and widely disseminate this information so that immediate and longer term disease prevention and control interventions can be implemented (IOM/NRC 2009)."

For influenza virus surveillance in swine in the United States, the rationale for a surveillance system includes not only protection of public health, but detection, discovery, and sharing of virus isolates to facilitate updates for vaccines, refine diagnostic assays, and determine the distribution of new influenza strains in swine to inform further policy decisions (USDA/APHIS 2009). In Europe, the Research Programme of the European Commission funded the coordination of the European Surveillance Network for Influenza in Pigs (ESNIP), a group that set out on a coordinated surveillance mission many years earlier, in 2001, with the stated goal of being to first standardize diagnostic techniques used for surveillance and detection of influenza viruses in pigs. Once the initial goals were achieved, the wealth of information from the surveillance efforts was leveraged for a second round of studies (ESNIP 2007) on the epidemiology and evolution of influenza viruses in European pigs and to optimize influenza diagnostic assays for swine (Kyriakis et al. 2010a). Naturally, a listed rationale for ESNIP 2 is, "to obtain insights into the public health risk of influenza in swine by monitoring swine for avian influenza viruses and by comparison of influenza viruses in swine and in human populations." ESNIP 3 has since been launched to "…increase the knowledge of the epidemiology and evolution of swine influenza virus in European pigs" with significant research investment directed toward detailed antigenic and genetic characterization of influenza virus strains isolated from pigs (European Commission 2010).

The surveillance programs for swine influenza in developed countries such as the United States and those of the European Union are striving to best coordinate efforts not only with multiple disciplines and agencies, but also with swine producers. While the benefits to human and public health are tangible in that understanding influenza virus patterns in swine may lead to more accurate and timely diagnosis of zoonotic influenza events, the reward for swine producers is less definable. The perception that influenza surveillance programs for pigs have fewer advantages for pork producers is likely due to several reasons, including the minimal impact of influenza virus infection on overall swine health and productivity, fear that trade and profits will be negatively affected, and the lack of readily available, consistently reliable, and inexpensive vaccines to control influenza once it is detected. Furthermore, the funding for such surveillance efforts in pigs requires commitment from all sectors, including animal agriculture, food production, human, and public health (AASV 2009). Securing funding and increasing participation in influenza surveillance programs for swine are challenges that need to be addressed.

1.3 Challenges to Surveillance in Animal Populations

From the outset, any effort to conduct influenza surveillance in pigs faces several unique challenges. For starters, respiratory disease is relatively common in pigs and the clinical signs and gross lesions associated with influenza virus in pigs are not entirely specific to influenza. While influenza virus is a significant disease in pigs, there is no official disease reporting requirement for influenza because clinical disease seldom leads to dramatic mortality or severe economic losses in a herd. Additionally, influenza virus is a highly mutagenic virus that can be exchanged among multiple species, with most concerning exchanges occurring between animals and humans. Due to the nature of the global economy, both humans and animals are increasingly mobile regionally and internationally, making comprehensive surveillance difficult across species and geographic boundaries. These factors pose challenges to any influenza surveillance effort in pigs and illustrate the importance of a coordinated surveillance approach.

The first challenge to swine influenza surveillance, and to any early warning system for swine infectious disease, is the inability to reliably detect disease through observations of clinical signs. The clinical signs associated with influenza virus in pigs are generally attributed to Porcine Respiratory Disease Complex (PRDC), a polymicrobial pneumonia caused by several common swine respiratory viruses and bacteria (Brockmeier et al. 2002; Straw et al. 2006). While it is true that influenza A viruses are frequently isolated from pigs with PRDC and evidence of exposure via serological assays is common in growing swine (Brockmeier et al. 2004), it would be wrong to ascribe all clinical signs of respiratory disease in a swine population to influenza without more discriminatory diagnostic methods. Therefore, while reliance on clinical signs and gross lesions for disease detection in pigs has proven to improve the sensitivity of disease detection for other swine diseases such as classical swine fever (Elbers et al. 2003), the sole use of clinical signs as a detection method can lack specificity (Engel et al. 2005). Even swine diseases with hallmark clinical signs, e.g. vesicular exanthema of swine, have cases that may be mild enough to fail detection by clinical observation alone (Schnurrenberger et al. 1987). Similarly, many uncomplicated influenza virus infections in pigs are also mild. It has been shown that single virus infections result in transient clinical signs (Van Reeth et al. 1996). Hence, clinical signs as a method of detection for influenza virus would also likely result in numerous missed cases as well as an abundance of false positive cases. Finally, using clinical signs to detect influenza requires observation by a trained veterinary professional or animal caretaker. Thus, it bears mentioning that surveillance methods, like direct clinical observation, requiring close contact with animals infected by with a potentially zoonotic disease can pose health risks to workers, another potential challenge (Myers et al. 2006; Bos et al. 2010).

The challenges associated with tracking “transboundary” viruses in animals, including influenza, have been reviewed previously (Domenech et al. 2006; Lynn et al. 2006; Gubernot et al. 2008) and the impact of human travel on respiratory

disease epidemics such as Severe Acute Respiratory Syndrome (SARS) in people has been examined extensively. However, the component of virus transmission from humans to pigs has not been a significant consideration other than in retrospective analysis of the 1918 Spanish Flu pandemic up until 2009 H1N1 pandemic (Hofshagen et al. 2009). Clearly the impact of human travel and the potential for infecting pigs with novel influenza viruses are evident now. Yet few surveillance systems that exist are capable of capturing both the human and animal data needed to shed light on the existing barriers that prevent or gateways that allow transmission to occur between species.

Migratory waterfowl represent another potential transmission source for influenza to pigs, as demonstrated experimentally (Kida et al. 1994) and naturally (Pensaert et al. 1981; Karasin et al. 2000). In the more recent report on natural infection of pigs with H4N6 influenza, waterfowl on a lake near a swine farm in Canada were implicated as the source of infection in pigs (Karasin et al. 2000). Even with confinement rearing of pigs, exposure to water-borne virus is possible in cases where surface water is used untreated as a water supply for the pigs. Pigs raised partially or completely outdoors could face a higher exposure risk. In the case of the H4N6 influenza virus infection, pigs were raised in confinement. The authors provide evidence of pig to pig transmission of the H4N6 influenza virus within the herd.

In contrast, the first widespread detection of H3N2 influenza virus in pigs in the United States in 1998 was followed by widespread dissemination of H3N2 throughout the North American swine population and subsequent reassortment with other influenza viruses (Ma et al. 2006). This is significant in light of the tremendous increase in movement of growing pigs throughout North America over the past 20 years, another significant challenge for surveillance of influenza in pigs. Data from the Minnesota Board of Animal Health on the movement of growing pigs and breeding swine into Minnesota are illustrative of this point (Fig. 1). There has been more than a seven fold increase in the number of feeder pigs imported into Minnesota over a 5-year period (fiscal years 1994–1999) and this number has doubled again in the subsequent 5-year period (fiscal years 1999–2004), with shipments originating from Canada and 31 other U.S. states (source: Minnesota Board of Animal Health). This movement of pigs at young ages (3–11 weeks) provides a source of pigs that are potentially infectious or susceptible (or both) to particular influenza virus strains. This extent of interstate and international movement is an important consideration when designing surveillance methods for influenza in pigs.

Finally, there is a potential challenge to influenza surveillance in pigs if producers are reluctant to participate in such a program. Diagnostic testing costs can be a barrier to surveillance particularly during protracted periods of unprofitable production such as occurred in 2009 in North America. Producers and veterinarians may also be reluctant to participate in surveillance programs that are perceived to have a potential negative impact on marketability of pigs from a specific site or more generally for the marketing of pork.

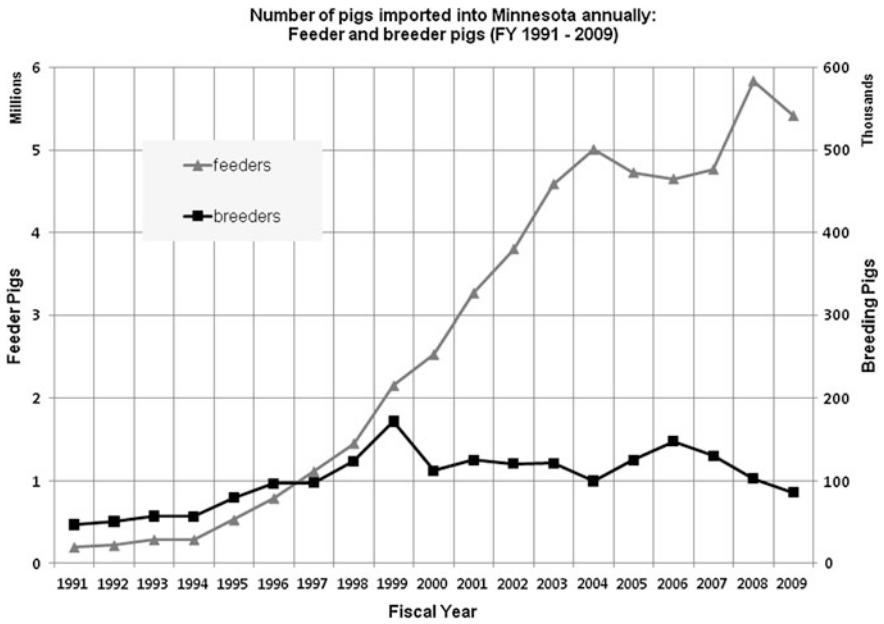

Fig. 1 Number of growing and adult pigs imported into Minnesota annually during fiscal years (FY) 1991 and 2009 according to the Minnesota Board of Animal Health. *Triangles* represent growing (feeder) pigs. *Squares* represent adult (breeder) pigs

1.4 Surveillance Design and Logistics

Surveillance design parameters depend on the objectives of the surveillance program as outlined previously. For example, surveillance parameters would be different if the objective is to identify the most prevalent influenza virus subtypes in pigs in a particular region versus whether influenza virus has been eliminated from a specific swine herd (Torremorell et al. 2009). Designing a surveillance program also requires a thorough understanding of the behavior of the virus in pigs, available diagnostic tests, and the production practices used for raising pigs that are to be monitored. Important features of influenza virus infections in pigs are illustrated in Fig. 2 and discussed in detail in other chapters (Clinicopathological Features of Swine Influenza) in this text.

Briefly, it is critical to remember that pigs develop a fever and begin shedding virus rapidly following exposure to influenza virus. Peak virus excretion follows the peak of fever very closely and declines rapidly thereafter. Circulating antibodies are detected within 10–14 days of infection. On an individual pig basis, there is a window of time following infection in which the virus has been cleared, antibodies have not developed, and the pig appears not infected.

Surveillance design is also a function of the tests available for use. Tests intended to detect virus need to be applied during the first week following infection,

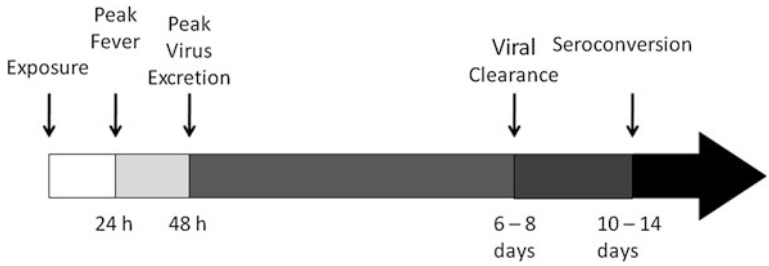

Fig. 2 The dynamics of influenza virus infection in swine represented by the simple timeline here are useful for designing surveillance testing protocols

preferably on samples from pigs that are still febrile. Serological tests such as hemagglutination inhibition can be used to evaluate samples before and after the expected time of seroconversion to specific subtypes of influenza. Serological tests are also available in an ELISA format that detect antibodies against all influenza A subtypes (Ciacci-Zanella et al. 2010) or certain individual subtypes. Influenza diagnostic assays for both antigen and antibody detection are discussed in detail in Sect. 2. Once established diagnostic assays are chosen for the surveillance program, the next critical component is a proper specimen selection and sampling strategy.

Specimen selection and sampling strategies. The specimen of choice within a surveillance program again relates to the objectives of the surveillance as well as the availability of appropriate samples for collection and testing. A variety of specimens are suitable for SIV detection in pigs, including nasal swabs, tracheal swabs, tracheal fluid, lung lavage fluid, and lung sections. For ante-mortem diagnosis of SIV, nasal swabs are one of the more easily obtainable samples. Oral fluids collected from pigs on a group basis represent an alternative to nasal or oropharyngeal swabs. Oral fluids have been used extensively for diagnostic tests in human medicine and are now being applied in swine herds for detecting pathogens and antibodies against the pathogens (Prickett and Zimmerman 2010). Specific applications of oral fluids for influenza virus testing are discussed in Sect. 2.2. Additionally, testing air samples for the presence of swine influenza virus is in the early stages of development (Hermann et al. 2006) and could find application in broader surveillance applications. Postmortem examinations of pigs infected with influenza A viruses have detected the virus (Vincent et al. 2009a; Yazawa et al. 2004) primarily in respiratory tract tissues (nasal turbinates, trachea, and lung), but also in tonsil and bronchial lymph node. The sites for virus replication are similar for historical isolates of "classical" H1N1 swine influenza virus (Yazawa et al. 2004) and 2009 pandemic H1N1 (Vincent et al. 2009a). Postmortem tissues are considered ideal specimens as they can also be examined for gross and microscopic lesions. Thus, complete necropsies with histopathological examinations can further our understanding of the pathogenesis of influenza A viruses in swine. Regardless of the specimen collected, the sample size chosen from the population of concern will affect successful detection of influenza in swine.

Sample size determination for surveillance programs is a function of what test is to be employed and how *prevalent* the target organism is within the population. In other words, the number of pigs shedding influenza virus at the time of sampling is likely to be different from the number of pigs with serum antibodies depending on when the pigs are sampled. The calculation of an adequate sample size required is fairly straightforward once all the other elements of the sampling frame are established, i.e. sensitivity and specificity of the test, prevalence of the target organism within the population, population size to be sampled, and desired confidence in the end result.

A formula that has been used extensively in swine disease surveillance programs for many years is given below:

$$n = (1 - (1 - \alpha)1/d)(N - (d/2)) + 1$$

where N is the population size, d is the number of positives in the population (expected or threshold prevalence for detection), α is the desired confidence level, and n is the number needed for testing (Cannon and Roe 1982). For example, if one assumes that a diagnostic assay is 100 % sensitive and specific, a sample size of $n = 30$ from a herd of infinite population N, will provide a 95 % confidence level of detection of disease if the disease prevalence is 10 %. In most situations, the diagnostic assays employed are not 100 % sensitive. As sensitivity decreases, the sample size n must increase.

Databases and sharing of information. Our experience with swine influenza databases indicates that populating the database and sharing the information is most successful when the information is used for a specific and important purpose. Testing for swine influenza virus is a regular activity at veterinary diagnostic laboratories. Serology results are generally used for making decisions on vaccine timing and are not typically collated into common databases. Virus detection by PCR and virus isolation is used to determine the role of influenza virus in clinical disease. Viruses isolated from clinical cases are often used for the production of autogenous vaccines based on the results of virus sequencing information. Virus sequencing information is often assembled into dendrograms to follow virus trends over time and geography. Each piece of the collective diagnostic information has a role in influenza diagnosis and control on a herd or production system basis. By definition, this brings the maintenance of the database close to the end user, who also happen to provide the data inputs.

Financial incentives, such as third party payment for sequencing information, have not appeared to be as important as the direct need for information in terms of motivating producers to participate in surveillance programs up to now. The degree to which producers and veterinarians are interested in sharing disease surveillance information among groups is promising but has not yet been fully determined (Davies et al. 2007).

1.5 Examples of Influenza Surveillance in Swine

Comprehensive surveillance programs are needed to detect new influenza strains especially the ones with pandemic potential so we can increase our preparedness to it. Effective surveillance programs should include detection of influenza viruses in

humans and animals including pigs. It should also include detection of viruses distributed throughout the world particularly in high risk areas where humans, poultry, and pigs coexist. Surveillance in pigs is considered crucial because pigs have receptors for human, swine, and avian influenza viruses potentially favoring the arising of new viral reassortants. Unfortunately such a global comprehensive surveillance program has not been put in place yet but attempts have been made at the local and regional levels. One limitation of this approach is that the information is not always integrated and shared across species and regions diminishing the effectiveness of comprehensive surveillance efforts.

Detection of influenza viruses in other mammalian species such as cats, dogs, bovine, and equine should also be considered as part of the integrated programs. Although a coordinated global surveillance initiative in pigs does not exist yet, there are examples of programs that over the years have provided a significant but incomplete picture of the circulating influenza viruses in pigs. In addition, the programs that are being planned to actively collect data and specimens for influenza will help to bridge the current gap in influenza surveillance in pigs.

Serosurveillance of pigs in North America. In the US, surveillance studies using serological methods have been based on the sampling of pigs at the point of slaughter and the testing of samples submitted to veterinary diagnostic laboratories. In these studies, pigs originated from various Midwestern States and were representative of pigs owned by multiple enterprises throughout the US. This was the method of choice for many years when other methods of sampling were not available.

In the US, several serological surveys have been conducted. It was demonstrated during 1976/1977 (Hinshaw et al. 1978) and 1988/1989 (Chambers et al. 1991) that influenza virus infections were common among pigs. The percentage of pigs seropositive against classical swine H1N1 viruses ranged from 20 to 47 % in 1976/1977 and 51 % in 1988/1989. In contrast serologic evidence of H3 virus exposure was remarkably lower in both studies (1.4 % in 1976/1977 and 1.1 % in 1988/1989).

In a subsequent study conducted in 1997/1998 (Olsen et al. 2000), 27.7 % of pigs were seropositive to swine H1 virus, 8 % to an H3 human virus, and 7.6 % to an H1 avian virus. These results indicated that pigs were exposed to human H3 and avian viruses to a greater extent than in the past. The finding that the study population tested positive to human H3 influenza virus was of particular significance. Up to that point, detection of H3-subtype influenza viruses in US pigs was rare although it was detected regularly among pigs in Asia and Europe. The findings in 1998 indicated a dramatic pattern change for influenza epidemiology in North America. A Canadian study indicated that seroprevalence to H3N2 viruses in 2002 was negligible although seroprevalence to H1N1 remained high (24.3–61.1 %) (Poliak et al. 2008).

Therefore, influenza surveillance using serological methods has provided useful information in the past but its use has become less reliable due to the broader use of influenza vaccines in pigs and the inability to differentiate antibodies induced by vaccine strains from field strains. In addition, serological methods may not always be able to differentiate infection by strains within a subtype or even between subtypes. The limitations of serological assays are discussed in further detail in Sect. 2.3. For these

reasons, virus molecular characterization methods have become widely used and are better able to detect genetic differences among viruses.

Surveillance provided by U.S. Veterinary Diagnostic Laboratories. State and private diagnostic laboratories in the US constitute a rich resource of samples and data for influenza virus surveillance in pigs. Thousands of cases are submitted to the diagnostic laboratories by practitioners and producers to investigate respiratory disease. Most of the cases originate from US herds but may include samples from Canadian herds and a few countries located in Central and Latin America. In many of the cases submitted, influenza virus is detected and diagnosed. As an example, more than 4862 influenza A viruses have been isolated from swine respiratory specimens at the University of Minnesota Veterinary Diagnostic Laboratory (UMVDL) between January 1, 2001 and June 1, 2010 (Gramer and Torrison 2010). In addition 200–700 influenza A virus nucleic acid detection tests (RT-PCR) are conducted monthly on swine respiratory specimens submitted to the UMVDL. The detection of influenza A virus by PCR is followed by subtyping and even partial hemagglutinin gene sequencing when funding is available. Because of confidentiality issues, data derived from these diagnostics is reported solely to the submitting veterinarian and animal owner. While some of the data is shared with the influenza research community, the majority is not automatically released to any publically accessible surveillance databases. Rectifying this situation is not straightforward, but would likely involve discontinuing the institutional practice of considering animal influenza virus isolates as the *intellectual property* of the owner or researcher and assuring anonymity and prevention of penalties to clients submitting specimens. Nevertheless, diagnostic laboratory data do constitute a valuable resource. In the US, the data generated represent the types of influenza viruses circulating in domestic swine and has resulted in vaccine strain updates and diagnostic reagent revision.

It can be argued that surveillance conducted through routine submissions to diagnostic laboratories is passive, syndromic, and retrospective producing only partial analysis of viruses. Whole genome sequencing of influenza A virus isolates from pigs is needed to detect virus changes and reassortment events that may result in new strains of pandemic potential (Vijaykrishna et al. 2010). Efforts, such as that of the USDA, are designed to integrate the US veterinary diagnostic laboratory network influenza detection and characterization into a more integrated and comprehensive surveillance plan (USDA/APHIS 2009).

Passive/Syndromic surveillance programs. During the last few years discussions have taken place in the US to have an active surveillance influenza program in pigs similar to those for people and poultry for detecting high pathogenic avian influenza or detecting strains of clinical importance. Such a program has not yet been fully possible in pigs although tremendous advances have been made. As a result of the 2009 pandemic, the USDA in cooperation with the CDC and industry allies initiated a voluntary influenza surveillance program in pigs (USDA/APHIS 2009). Although participation in the program has been limited, pork producer, and veterinarian involvement is slowly increasing and contributions of specimens for virus isolation to the surveillance efforts are on the rise.

In addition, influenza is proposed to be part of a comprehensive and integrated surveillance program being designed to protect the US food supply from the impact of diseases considered exotic in the US (AASV 2010). This program has many goals including actively testing for foot-and-mouth disease, classical swine fever, *Brucella suis*, Aujeszky's disease, *Trichinella spiralis*, *Toxoplasma gondii,* and influenza A virus. Many stakeholders are participating in the design of this program, including the USDA, HHS/CDC, National Pork Board, National Pork Producers Council, American Association of Swine Veterinarians, Veterinary Diagnostic Laboratories, State Animal Health Officials, and State Pork Producer Associations. In regards to influenza, the program aims to determine the prevalence and variety of influenza viruses in US swine, facilitate influenza strain selection for vaccine production, provide continuous improvement of diagnostic testing capabilities, and warrant anonymity to the submitting systems to facilitate cooperation. Such a system should facilitate the cooperation and sharing of information and specimens among stakeholders.

Hong Kong surveillance program for influenza in slaughtered swine. For over a decade, researchers at the University of Hong Kong have participated in an internationally funded, systematic, virological, surveillance program for influenza A viruses in swine slaughtered at one abbatoir in Hong Kong (Vijaykrishna et al. 2010). A majority of the swine slaughtered at this abbatoir are said to originate from mainland China. Routine visits are made to the abbatoir wherein nasal or tracheal swabs from slaughtered pigs are collected, subjected to virus isolation via inoculation in eggs or MDCK cells, and then characterized by hemagglutination inhibition (HI) and sequencing. This slaughter surveillance program has yielded interesting information regarding the genetic constellation of viruses present in China and Hong Kong (Smith et al. 2009; Peiris et al. 2001).

Research-based surveillance. In an effort to bridge the gap on influenza surveillance in pigs, the United States National Institutes of Health funded Centers of Excellence for Influenza Research and Surveillance (CEIRS) have directed some of their research efforts toward active influenza surveillance in swine-dense areas in the Midwestern United States (NIAID 2010). The information from an active surveillance program such as this is sorely needed as growing swine are more representative of the population of pigs most likely to be infected with influenza A virus (Brown 2000), and, because the epidemiology of the virus in swine farms is not well understood (Olsen et al. 2006), an active surveillance program can shed key information on the epidemiology of influenza in swine. In the NIAID sponsored program on active influenza surveillance in swine, thirty nasal swabs are collected every month for 12 consecutive months from growing pigs in 34 separate farms. Swabs are tested for influenza virus by PCR and virus isolation. During collection, the age of the pigs, group clinical signs, and influenza vaccination history are recorded. Farm characteristics, such as herd size, building design, proximity to other farms, biosecurity practices, are also recorded in an attempt to determine possible risk factors associated influenza virus infection. Data on pig age, clinical status, meteorological, and environmental conditions are collected to obtain information on current influenza isolates, their distribution, and disease characteristics.

Summary of international surveillance programs. In Europe, the Research Programme of the European Commission funded the coordination of the European Surveillance Network for Influenza in Pigs (ESNIP). This group became active in 2001 and continues the efforts to increase the knowledge of the epidemiology and evolution of swine influenza virus in European pigs.

In Hong Kong, the surveillance program consists of the isolation of influenza virus at the point of slaughter. Throughout this program a limited but significant number of viral isolates has become available representing the only active systematic influenza surveillance program in the world.

In South and Central America, formal surveillance efforts are nonexistent and are complicated by the fact that some countries consider influenza in pigs an exotic disease limiting the ability to even conduct routine influenza diagnostics.

2 Diagnostics for Swine Influenza

Diagnosis of swine influenza in the twenty-first century has become more complicated due to the presence of multiple strains of influenza viruses cocirculating in pigs (Webby et al. 2004). Due to the introduction of these multiple strains, the diagnosis and characterization, it is important to understand the many tests that are being used to better characterize influenza virus infections in swine.

2.1 Clinicopathology

Clinical signs and characteristic macroscopic and microscopic lesions are useful in making a presumptive, but not definitive, diagnosis of swine influenza infection (see the chapter regarding Clinicopathological Features of Swine Influenza in this text and also Sect. 1.3 and Fig. 2). Laboratory detection of the whole virus, viral antigen, viral nucleic acids or anti-viral antibodies within tissues, serum or other clinical samples is needed for definitive diagnosis.

2.2 Direct Detection Methods

2.2.1 Detection of Influenza Virus Antigen

Immunohistochemistry (IHC) and immunofluorescence (IFA) are used to detect influenza virus antigen in frozen or formalin-fixed tissues using different antibodies (Guarner et al. 2000; Haines et al. 1993; Larochelle et al. 1994; Onno et al. 1990; Vincent et al. 1997). The nucleoprotein (NP) is well-conserved among influenza A viruses; therefore, anti-NP antibodies can be used to detect all

subtypes of influenza A viruses. However, the hemagglutinin (HA) protein is subtype-specific and hence is used to detect specific subtypes of influenza virus. The NP antigen is located in the nucleus and cytoplasm of infected cells (Guarner et al. 2000; Haines et al. 1993; Larochelle et al. 1994; Vincent et al. 1997) while the HA is located in the cytoplasm and along the cell surface (Guarner et al. 2000).

Direct immunostaining methods use antibodies that are labeled with biotin, fluorophore, enzyme, or colloidal gold (Buchwalow et al. 2010). Although technically difficult and time-consuming, indirect immunostaining methods have higher sensitivity and are more commonly used for diagnostic tests (Buchwalow et al. 2010). These methods use an unlabeled primary antibody followed by a labeled secondary antibody. The application of the substrate then results in amplification of the colorimetric signal produced by the enzyme attached to the secondary antibody (Buchwalow et al. 2010). Of the indirect methods, the standard avidin–biotin complex (ABC) method of IHC has been widely used for SIV detection (Haines et al. 1993; Vincent et al. 1997). However, with this method there can be background staining due to endogenous biotin in the tissues (Vosse et al. 2007). Therefore, these methods have been adapted to polymer-based IHC method (Richt et al. 2006) that uses a polymer backbone on the secondary antibody to attach to the enzyme instead of avidin–biotin complex (Sabattini et al. 1998).

A number of rapid immunoassays, most being enzyme-linked immunosorbent assay (ELISA)-based tests kits are commercially available that can detect influenza virus antigen in clinical samples. Most of these tests have been developed specifically for human and avian applications and the viral proteins that are detected by these kits are HA, neuraminidase (NA), or NP. Five of the kits licensed for human application were found to have sensitivity of 67–71 % and specificity of 99–100 % for Influenza A (Hurt et al. 2007). The sensitivity was higher for specimens containing more than 10^5 copies/ml of influenza virus RNA as determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (Cheng et al. 2009) or 10^3–10^5 $TCID_{50}$/ml of virus as determined by virus titration in cell cultures (Chan et al. 2009; Hurt et al. 2009). For avian samples, in which sensitivity of RT-PCR is known to be lower than that of virus isolation in embryonated chicken eggs, the sensitivity of antigen detection kits was comparable to that of RT-PCR (Cattoli et al. 2004); the minimum amount of virus needed was 5×10^4 $TCID_{50}$/ml (Fedorko et al. 2006).

2.2.2 Detection of Nucleic Acids

First described in 1985 (Saiki et al. 1985), the polymerase chain reaction (PCR) has been used to clone DNA, sequence, and analyze genes, identify people by their unique genetic fingerprint and diagnose infectious and genetic diseases. The production of complementary DNA (cDNA) from RNA was made possible by the development of RT-PCR. In 1992, PCR was made even more powerful with the innovation of real-time PCR (RRT-PCR) (Higuchi et al. 1992). Although semiquantitative in nature (Kubista et al. 2006), several RRT-PCR testing

protocols have been developed for the detection and quantitation of influenza A viruses including SIVs (Spackman et al. 2002; Spackman and Suarez 2008).

The use of RNA extraction and purification methods varies by the type of sample being tested. For example, RNA can be extracted directly from infected amnioallantoic fluids, cell culture supernatants, bronchoalveolar lavage fluids (BALF), and oral fluids. However, for certain clinical diagnostic samples, prior processing is necessary. Tissue samples, such as lungs, are first made into a 10 % w/v homogenate using a balanced salt solution or a viral culture medium while nasal swabs are usually suspended and vortexed in a test tube with 2 ml of the above media. Although labor-intensive, standard organic extraction procedures produce high purity RNA from most any sample, including tissue homogenates, paraffin-embedded tissues, and body fluids (Sun 2010). However, commercial kits that use magnetic beads or solid-phase adsorption are more sensitive and easy to use with consistent results (Sun 2010). Commercial kits, such as RNeasy and QIAamp RNA kits (Qiagen, Valencia, CA) and PureLink™ RNA kit (Invitrogen, Carlsbad, CA) are based on solid-phase adsorption using silica-membrane spin columns. Commercial kits for magnetic bead extraction, such as MagMAX™ (Applied Biosystems, Foster City, CA) and EZ1 (Qiagen, Valencia, CA) are useful for liquid samples that have low virus concentration or contain PCR inhibitors, such as oral fluids, semen, urine, feces, and blood (Chan and McNally 2008; Das et al. 2009).

To detect a broad range of influenza A subtypes, primers for RRT-PCR are designed to target the conserved matrix (M) or nucleoprotein (NP) genes. The USDA-validated avian influenza RRT-PCR for the M gene (Spackman et al. 2002; Spackman and Suarez 2008) has been adapted for the detection of SIV in swine samples. The minimum detectable concentration of the virus for this procedure ranges from 10^{-1} to 10^{1} $TCID_{50}$/ml depending on the virus strain (Landolt et al. 2005; Richt et al. 2004). While virus isolation is still the gold standard test for influenza viruses, RT-PCR is an accurate, rapid, and sensitive technique that can be used to screen a large number of samples in a short period of time. The main disadvantage of RT-PCR is that it detects only the viral RNA and does not determine whether virus is viable or not. Since virus isolation depends on sample inoculation in a live culture system and detects the presence of live virus, it is often used in conjunction with RT-PCR to verify the presence of viable virus.

2.2.3 Detection of Whole Virus

Egg inoculation (EI) using nine to eleven-day-old embryonated chicken eggs is considered the gold standard for isolation and propagation of avian influenza viruses and certain egg-adapted SIVs (Clavijo et al. 2002; Swenson et al. 2001). However, it has been demonstrated that human influenza viruses propagated in chicken embryos acquired amino acid changes in their HA gene resulting in antigenic variation of the virus (Katz et al. 1987; Katz and Webster 1992; Meyer et al. 1993; Robertson et al. 1995). Comparatively, there was little to no genetic or

antigenic variation in the same viruses when propagated in mammalian cell lines (Katz et al. 1987, 1990; Katz and Webster 1992; Meyer et al. 1993; Robertson et al. 1995), including Vero, MRC-5, BHK-21, and fetal porcine kidney cells. Of these, the Madin-Darby canine kidney (MDCK) cells have the highest sensitivity and are most commonly used in research and diagnostic applications (Meguro et al. 1979). For maximum sensitivity, inoculation of chicken embryos and/or another cell line is recommended in addition to MDCK cells.

Sample preparation for virus culture is the same as described for RT-PCR (Meguro et al. 1979). Influenza A viruses may replicate in cell cultures within 24–48 h or may take up to 5–6 days if the initial virus concentration in the sample is low. Growth of virus in cell cultures induces the production of cell lysis or cytopathic effects (CPE). Often a second blind passage is necessary for certain strains to show CPE. Once the virus has grown in cell cultures, tests can be performed on the culture supernatant to confirm viral identity. Although not a definitive assay, hemagglutination (HA) of chicken erythrocytes can be taken as a presumptive diagnosis of the virus and for approximation of the amount of virus present in the cell culture supernatant (1 HA unit approximates 5–6 $\log_{10}$ of virus). A more accurate method of quantifying virus is virus titration by inoculation of a set of serial dilutions in cell cultures (Villegas and Alvarado 2008). For definitive virus identification, the culture supernatant can be tested by RT-PCR or commercial influenza antigen test kits based on NP or M antigen. Since virus culture usually contains higher concentrations of virus than the original sample, sensitivity issue seen with clinical samples is usually not a problem when using antigen test kits.

Although virus isolation requires specialized equipment and maintenance of cell cultures and/or embryonated eggs, it is a standardized procedure that is available in most diagnostic laboratories. The virus isolated in cell culture can be cryogenically preserved for years and used for further characterization and vaccine production.

2.3 Indirect Detection

Although the clinical signs of influenza infection coincide with the presence of virus in nasal secretions, the isolation of virus by the gold standard method of virus culture or its detection by RT-PCR can be difficult when the period of virus shedding is brief. It has been found in vaccine challenge studies that shedding can be as transient as 24–72 h (Heinen et al. 2001, 2002; Van Reeth et al. 2001, 2003, 2006).

In situations when influenza virus is suspected but no longer detectable at the time of testing, detection of specific immunoglobulins may be undertaken. Immunoglobulins (predominantly IgG) are formed in swine at detectable levels within 1–2 weeks post infection and peak at 4–7 weeks (Olsen et al. 2006). For this reason, it has been recommended that serum samples be collected from pigs at the time of infection and at 3–4 weeks after the onset of clinical signs to compare the acute versus convalescent response (Rossow et al. 2003). Since influenza

antibodies can be formed in response to both vaccination and exposure status, the interpretation of serologic assays will depend on both the vaccination and exposure status of the animals being tested. The serologic tests used to detect and measure influenza antibodies include: hemagglutination inhibition, serum neutralization, and enzyme-linked immunosorbent assays.

Hemagglutination inhibition (*HI*). The agglutination of red blood cells (RBCs) is a natural reaction that occurs in the presence of HA protein on the surface of the virus. HA can be specifically inhibited by influenza antibody, which can be measured in an HI assay. Optimum HA and HI reactions in SIVs occur with turkey or chicken RBCs, which are used in standardized tests (OIE 2008). Before conducting HI tests, it is imperative to remove non-specific inhibitors of viral hemagglutination and naturally occurring agglutinins from the serum samples to be tested. Inhibitors can be removed by treatment with receptor destroying enzyme (RDE) from *Vibrio cholerae,* heat inactivation, kaolin, or potassium periodate. Similarly, non-specific agglutinins can be removed by pretreatment of serum samples with chicken or turkey RBCs (Boliar et al. 2006; Pedersen 2008a; Regula et al. 2000; Ryan-Poirier and Kawaoka 1991; Springer and Ansell 1958; Subbarao et al. 1992). RDE and heat inactivation at 56 °C are the methods currently recommended to remove inhibitors (OIE 2008).

For the HI test, serial two fold dilutions of the test serum (starting at 1:10 and ending at 1:640 or 1:1280) are prepared in 96-well microtiter plates followed by the addition of 4–8 HA units of a single subtype of influenza virus in all wells containing serum dilutions. Following incubation for an hour at room temperature, 0.5 % suspension of RBCs is added to each well. In the absence of specific antibody, the virus is uninhibited (unbound) and is free to bind to the RBCs resulting in hemagglutination. However, if antihemagglutinin antibodies are present in the serum, such as after exposure or vaccination, the antibodies will bind to the hemagglutinin protein on the surface of the influenza virus, thus *inhibiting* the virus' ability to agglutinate the RBCs. The reciprocal of the highest serum dilution that inhibits HA is considered to be the HI titer of that serum (Fig. 2). HI titers greater than or equal to 1:40 are usually considered to be protective (Hancock et al. 2009).

The HI test is considered a standard test for the detection of SIV antibody (Villegas and Alvarado 2008) but is somewhat subjective in nature and the results may vary because of operator subjectivity and also upon repeating the test. Also, since there is broad cross-reactivity among the α, β, and γ clusters of the H1 subtype of SIVs, a positive HI titer may indicate a virus related to the virus of exposure, but does not definitively identify it. However, homologous virus reactions are typically stronger than heterologous virus reactions, resulting in higher HI titers. The advantages of this test are that it is a standardized procedure that is inexpensive and easy to perform and the results are comparable to more complicated tests, such as serum neutralization (Leuwerke et al. 2008; Vincent et al. 2006, 2009a).

Serum neutralization (SN) or virus neutralization (VN). The SN test detects virus-specific neutralizing antibody present in a serum sample. Serial two fold dilutions of the serum and a known amount of SIV are preincubated and then

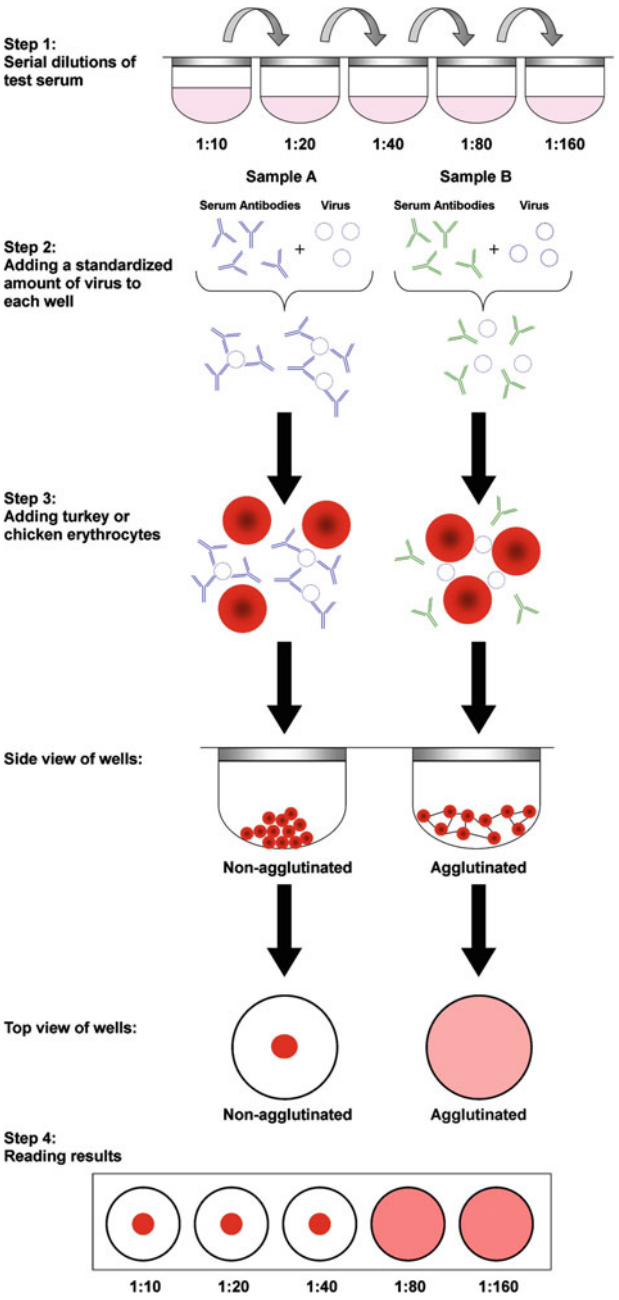

Fig. 3 Steps in a hemagglutination inhibition reaction. The antibodies on the left in sample A prevent the virus from agglutinating the erythrocytes. Whereas the antibodies on the right in sample B do not bind to the virus in step 2, which agglutinate the erythrocytes in step 3. The antibody titer shown in step 4 is read out as 1:40

added to MDCK cells to determine the highest dilution of serum that can neutralize virus infection of cells and production of CPE (Fig. 3). Neutralizing antibodies in serum sample block viral infection of cell culture and the virus is not available to produce CPE. However, if antibodies are not present, the virus is not blocked and is free to cause CPE in inoculated cell cultures. Reciprocal of the highest serum dilution that can neutralize virus infection is considered to be the SN titer of the serum. Since the test uses very small volumes of serum in cell monolayers contained in 96-well microtiter plates, it is often called micro neutralization. One of the advantages of SN over HI and enzyme-linked immunosorbent assays (ELISA) is that it demonstrates the biologic (neutralizing) activity of the antibodies present in the serum. Some of the disadvantages of this test are that it requires equipment and supplies used for virus cultures and the results can take up to 72 h to obtain. Also, the SN titers may vary when the test is repeated.

Enzyme-linked immunosorbent assay (*ELISA*). The ELISA test uses a 96-well plate that has been coated with influenza viral antigen. The serum sample is incubated in the coated wells for antibody attachment. After the unbound material is washed away, an anti-influenza monoclonal antibody that is conjugated to an enzyme is bound to the antigen. The unbound conjugate is washed away and the enzyme substrate (that produces a color change in the presence of the enzyme) is added to the wells. The color-changing reaction is stopped after 15 min and the amount of color produced is read as an optical density (O.D.) in a spectrophotometer (Fig. 4). The O.D. is inversely proportional to the amount of anti-influenza antibodies present in the test sample. Commercially available ELISA test kits include separate ELISA tests for H1N1 and H3N2 subtypes of SIV. Another ELISA that detects antibodies to a range of influenza A viruses is available and has been adapted for use in detecting anti-SIV antibodies (Ciacci-Zanella et al. 2010).

The commercial H1N1 ELISA uses an antigen prepared from a classical H1N1 SIV and, thus has a limited detection range of swine H1 subtypes. Although the H1N1 test is not designed to detect other influenza subtypes, it may sometimes cross-react with H3N2 because of some common epitopes between H1N1 and H3N2 viruses. In addition, the H1N1 test has been found to miss recently infected animals (Yoon et al. 2004). The H3N2 ELISA test was developed from a cluster I virus leading to lower reactivity with class IV viruses (Yoon et al. 2004). The MultiS-Screen ELISA (FlockChek^{TM}, Idexx, Westbrook, ME) uses a highly conserved epitope of influenza A nucleoprotein (NP) (Ciacci-Zanella et al. 2010). Preliminary studies indicate that this kit, while originally designed for use in avian species, also detects antibodies against subtypes common to swine (Ciacci-Zanella et al. 2010) (Fig. 5).

2.4 Virus Subtyping and Sequencing

Important for host range, antigenicity, and pathogenesis, the 16 HA and 9 NA genes are antigenically and genetically divergent and these variations are used for subtyping the influenza viruses. The cultured viruses were traditionally subtyped

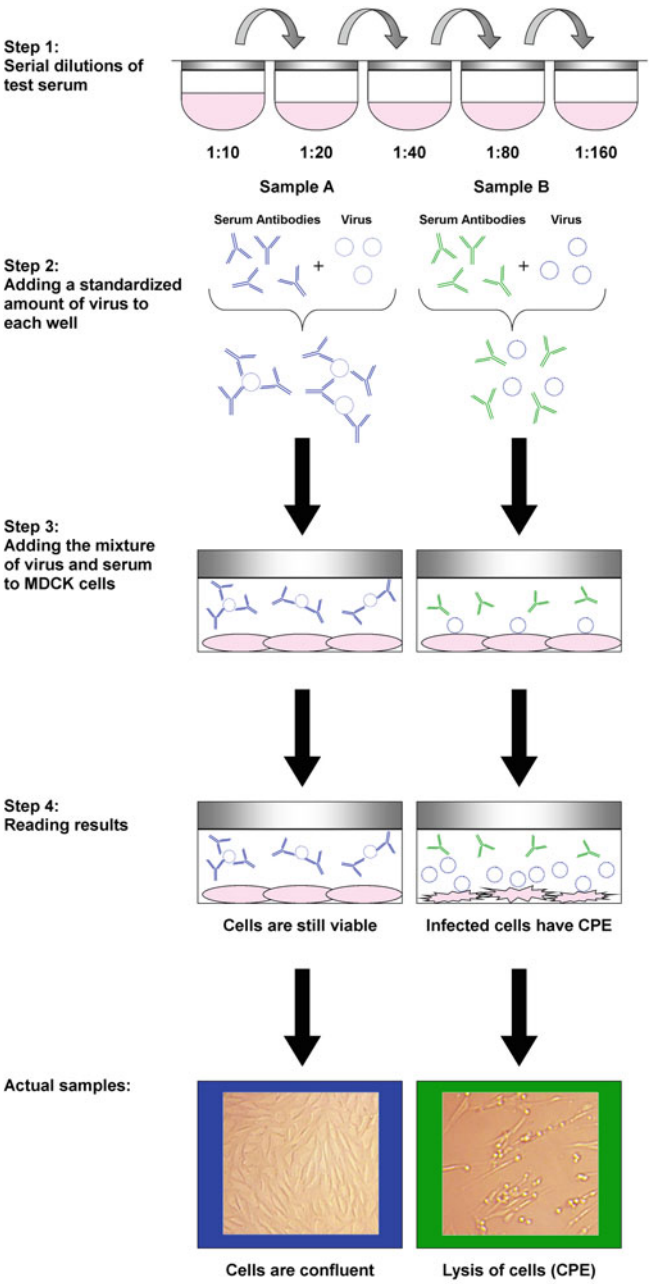

Fig. 4 Steps in a serum neutralization reaction. The antibodies in sample A on the left neutralized the virus in step 2. This resulted in no cytopathic effects (CPE) in step 4. Whereas the antibodies in sample B on the right did not neutralize the virus in step 2, resulting in infection of the MDCK cells and CPE in step 4

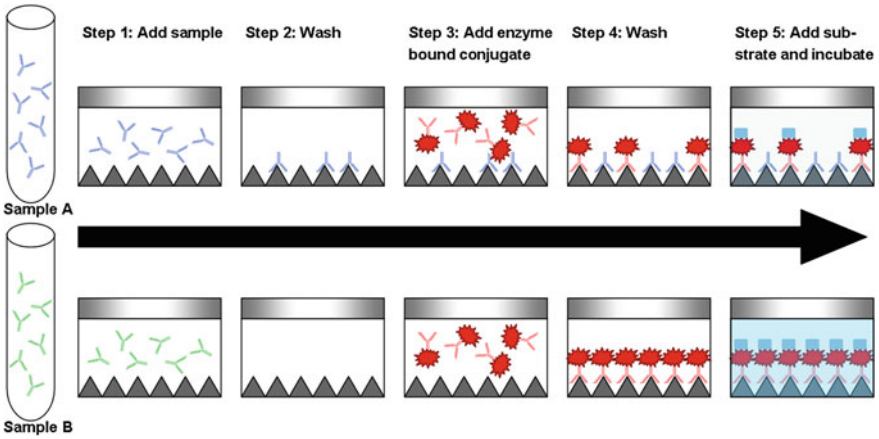

Fig. 5 Steps in a blocking ELISA test. The optical density of sample A is lower than sample B because the influenza A antibody in sample A is bound to the antigen coated on the bottom of the well, partially blocking the binding of the enzyme bound conjugate. The antibodies in sample B did not bind to the antigen and were therefore washed out in step 2. Figure adapted from http://www.idexx.com/pubwebresources/pdf/en_us/livestock-poultry/0965846.pdf

using HI and NA inhibition (NI) assays (Pedersen 2008a, b). The NI assay uses a dilution of the cultured virus between 1:4 and 1:32, depending on the virus concentration. There are several steps that include standardized NA antisera (N1–N9), fetuin, periodate, sodium arsenite, and thiobarbituric acid which result in a dark color if there is no inhibition and a light color if there is inhibition; the NA subtype has the light color result. Both of these assays are time-consuming and require standardized NA and HA antisera, which are often difficult to acquire. Therefore, RT-PCR is now regularly used for subtyping. Currently, HA and NA specific primers can be used for both detection and subtyping of influenza A viruses. Additionally, a number of multiplex and nested RT-PCR have been developed for subtyping with and without simultaneous detection of influenza A virus (Chander et al. 2010; Fereidouni et al. 2009; He et al. 2009; Lam et al. 2007; Li et al. 2001; Stockton et al. 1998; Yang et al. 2010).

In addition to subtyping, RT-PCR can also be used for sequencing all eight gene segments of influenza virus (Chander et al. 2010; Jindal et al. 2009). The sequences can be examined and compared to other sequences with molecular analysis tools; uncovering the evolutionary; and geographic relationships of influenza viruses. However, the amount of RNA in clinical samples is usually low compared to the other cellular materials and contaminating bacteria (Spackman and Suarez 2008). Therefore, cell culture supernatants and amnio-allantoic fluid containing a large concentration of whole virus, are recommended for sequencing and other molecular analyses (Spackman and Suarez 2008).

2.5 Limitations of Diagnostic Assays

The rapid evolution of influenza A viruses over the last decade has led to genetic and antigenic variation of the virus in North American swine. This has led to limitations in cross-reactivity for the serologic assays. These changes need to be kept in mind when interpreting the results of these tests. Although there is some antigenic cross-reactivity among the classical and reassorted α, β, and γ clusters of the swine H1 subtype, there is little to no cross-reactivity between these three clusters and the human-like δ cluster (Vincent et al. 2006, 2009b). This variability in the antigenic cross-reactivity was demonstrated in 2009 pandemic H1N1 virus for both North American and European swine H1 subtypes using sera from experimentally infected and vaccinated pigs (Kyriakis et al. 2010b; Vincent et al. 2010). The human-like viruses in the SwH1δ cluster were recently found to have two distinct antigenically divergent groups, which could result in additional limitations for serologic assays (Vincent et al. 2009a). Similarly among the swine H3 viruses, there is little to no cross-reactivity between groups I and IV. There is also limited to no cross-reactivity between swine subtypes, which means that multiple viruses from each subtype need to be tested to determine the subtype of the virus that produced the antibodies. To overcome the limitations of cross-reactivity and broaden influenza surveillance, the samples may first be screened by the MultiS-Screen ELISA followed by more specific tests, such as SN and HI assays to determine the subtype of the virus of exposure.

As the influenza virus continues to evolve, the primers for RT-PCR for detection and subtyping need to be continually validated and updated. Current testing stratagems rely on conserved nucleotide sequences for the primers. However, the variability in the HA and NA genes in avian influenzas have resulted in the design of multiple *wobble primers* to detect one subtype of influenza A without cross-reactivity with other HA and NA subtypes (Sidoti et al. 2010; Starick et al. 2000; Suarez et al. 2007). The avian influenza primers can be used for subtyping influenza viruses from swine or new subtyping primers can be designed using published sequences (He et al. 2009; Huang et al. 2009; Lee et al. 2008; Nagarajan et al. 2010). New technologies, such as enzyme hybridization and microarray, are being used for subtyping of influenza viruses across species (avian, human and swine) and detection of specific influenza viruses like 2009 pandemic H1N1 (He et al. 2009; Huang et al. 2009).

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Contemporary Epidemiology of North American Lineage Triple Reassortant Influenza A Viruses in Pigs

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Abstract The 2009 pandemic H1N1 infection in humans has been one of the greatest concerns for public health in recent years. However, influenza in pigs is a zoonotic viral disease well-known to virologists for almost one century with the classical H1N1 subtype the only responsible agent for swine influenza in the United States for many decades. Swine influenza was first recognized clinically in pigs in the Midwestern U.S. in 1918 and since that time it has remained important to the swine industry throughout the world. Since 1998, however, the epidemiology of swine influenza changed dramatically. A number of emerging subtypes and genotypes have become established in the U.S. swine population. The ability of multiple influenza virus lineages to infect pigs is associated with the emergence of reassortant viruses with new genomic arrangements, and the introduction of the 2009 pandemic H1N1 from humans to swine represents a well-known example. The recent epidemiological data regarding the current state of influenza A virus subtypes circulating in the Canadian and American swine population is discussed in this review.

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Current Topics in Microbiology and Immunology (2011) 370: 113–131
DOI: 10.1007/82_2011_196

Published Online: 22 January 2012

Contents

1 Brief Introduction to Influenza A Viruses

Influenza is a zoonotic viral disease that represents a health and economic threat to both human and animals worldwide. Influenza A viruses are the most studied of the *Orthomyxoviridae* since they can infect a large variety of birds and mammals including humans, pigs, horses, domestic poultry, marine mammals, cats, dogs and wild carnivores (Webster 2002; Thiry 2007). Wild aquatic birds were shown to be an asymptomatic reservoir for most subtypes of influenza A viruses (Scholtissek 1978; Fouchier et al. 2005). Moreover, influenza A virus ecology is intricate due to the high number of possible reassortment events and cross-species jumps that lead to their evolution (Webster et al. 1992). The hemagglutinin (HA) and the neuraminidase (NA) proteins encoded by gene segments 4 and 6, respectively, play a key role in the influenza life cycle and represent the primary targets of the host humoral immune response (Skehel and Wiley 2000). The HA protein is the most important determinant of virulence and host specificity as it binds to sialic acid-containing cell surface receptors on host epithelial cells (Shinya et al. 2006; Nicholls et al. 2008; Ayora-Talavera et al. 2009; de Wit et al. 2010). The HA mediates virus binding to *N*-acetylneuraminic acid-2,3-galactose (2,3-sialic acid) or *N*-acetylneuraminic acid-2,6-galactose (2, 6-sialic acid) terminal residues on sialyloligosaccharides for avian and mammalian virus primary binding predilection, respectively (Rogers and Paulson 1983). However, receptor binding restriction has been shown to be more complicated than previously understood, with tissues from human, swine and Japanese quail expressing both 2,3- and 2,6-sialic acid receptor types (Ito et al. 1998; Suzuki et al. 2000; Shinya et al. 2006; Wan and Perez 2006). Additionally, glycan array analysis has demonstrated that avian and mammalian adapted flu viruses can have binding spillover to the opposing receptor linkage type and that different strains bind preferentially to novel structures (such as sulphated and sialylated glycans) (Stevens et al. 2006). The NA is responsible for cleaving terminal sialic acid residues from carbohydrate moieties on the

surfaces of the host cell and virus (Gottschalk 1957), thus assisting in virus cell entry by mucus degradation (Matrosovich et al. 2004) and the release and spread of progeny virions (Palese et al. 1974). The remaining six segments encode for the following structural and accessory proteins: PB2 (segment 1), PB1 (segment 2), PA (segment 3), NP (segment 5), M1 and M2 (segment 7), NS1 and NEP (segment 8) (Lamb and Krug 2007). Both HA and NA genes undergo two types of variation called antigenic drift and antigenic shift. Antigenic drift involves minor changes in the HA and NA due to polymerase errors during replication, whereas antigenic shift involves major changes in these molecules resulting from replacement of the entire gene segment as a consequence of reassortment events in the event that two (or more) unique viruses infect the same cell (Webster 1971). Based upon the major differences within the HA and NA proteins, 16 HA and 9 NA subtypes, naturally paired in different combinations, have been identified thus far (Webster et al. 1992; Rohm et al. 1996; Fouchier et al. 2005). Only a limited number of subtypes have been established in mammals. For example, only viruses of H1, H2, H3, N1 and N2 subtypes have circulated widely in the human population (Webster et al. 1992; Alexander and Brown 2000) and only H1, H3, N1 and N2 subtypes have been consistently isolated from pigs (Webster et al. 1992; Olsen 2002).

2 Influenza A Virus in Pigs

2.1 First Detection in the United States

Swine influenza was first recognized in pigs in the Midwestern U.S. in 1918 (Fig. 1) as a respiratory disease that coincided with the human pandemic known as the Spanish flu (Koen 1919). Since then, it has become an important disease to the swine industry throughout the world. The first influenza virus was isolated in 1930 by Shope (1931) and was demonstrated to cause respiratory disease in swine that was similar to human influenza. This strain was subsequently recognized as an H1N1 influenza virus, and swine were utilized in the following years as a model to study influenza pathogenesis in a natural host.

2.2 Introduction of the Triple Reassortant H3N2 in Pigs

Among the RNA viruses affecting mammals, influenza viruses and coronaviruses represent, as a consequence of different molecular mechanisms, two of the best examples of viruses with exceptionally plastic genomes. Thus, we should not be surprised that the high mutation and reassortment rates have propelled the evolution of influenza viruses in pigs in recent years. However, from the first characterization of swine influenza virus until the late 1990s, the classical swine lineage H1N1 (cH1N1) was relatively stable at the genetic and antigenic levels in

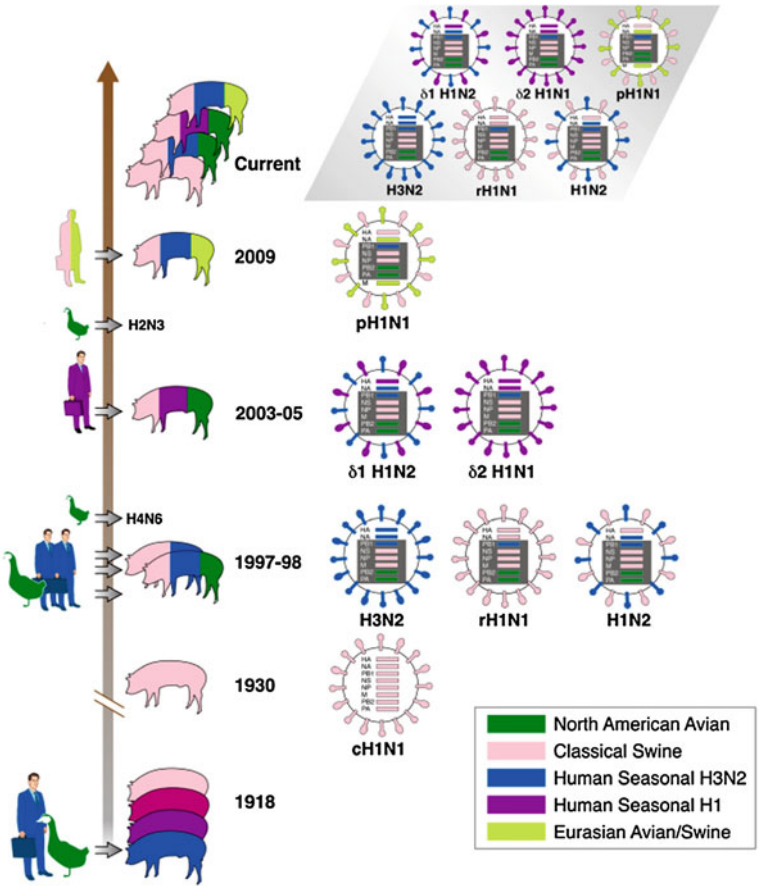


Fig. 1 Epidemiology and genetic composition of influenza viruses from U.S. and Canadian pigs. Swine virus lineage is color coded *pink*, avian lineage is coded *green*, human lineage is coded *blue* or *purple*. The chronology of transmission events leading to reassortant viruses with genes from swine, human and avian influenza virus lineages is visualized by the *vertical arrow*. The "Spanish flu" virus was transmitted from avian/human origin to pigs and evolved into the cH1N1, as indicated by the transition in color of pigs from *blue* to *light blue* to *red* to *pink*. The human and avian images to the left of the *vertical timeline* represent the species origin of viral gene segments donated to give rise to the swine influenza virus reassortants listed on the right side of the *vertical timeline*. *Time line* is not drawn to scale. Each viral subtype is represented with its eight gene segment arrangement. The triple reassortant H3N2 reassorted with the cH1N1 to produce rH1N1 and H1N2 subtypes with the triple reassortant internal gene (TRIG) cassette. Further reassortment events with two independent human H1 subtype viruses led to the δ1 H1N2 and δ2 H1N1. The source of the reassortment event producing the combination of gene segments in the 2009 pandemic H1N1 prior to its emergence in human and subsequent transmission from humans to pigs in 2009 is currently unknown. *Light green* indicates the Eurasian avian/swine lineage. The *gray* highlighted area illustrates the currently circulating influenza A subtypes in Canadian and American pigs

U.S. swine. Based on phylogenetic analysis, the cH1N1 lineage is closely related to the 1918 H1N1 Spanish flu virus (Easterday and van Reeth 1999) and other human influenza viruses isolated in the 1930s following the discovery of SIV. Although the cH1N1 was the predominant subtype causing disease in pigs until the late 1990s, there was serological evidence that human subtype H3 influenza viruses were circulating at a low frequency in U.S. pigs, but failed to establish a lineage with sustained transmission among swine (Chambers et al. 1991).

The epidemiology of influenza in pigs dramatically changed after the events of 1997–1998 (Fig. 1). In 1998, a severe influenza-like disease was observed in pigs in North Carolina with additional outbreaks in swine herds in Minnesota, Iowa and Texas. The causative agents for these outbreaks were identified as influenza A viruses of the H3N2 subtype. Genetic analysis of these H3N2 viruses showed that at least two different genotypes were present. The initial North Carolina isolate was a double reassortant and contained gene segments similar to those of the classical swine lineage (PB2, PA, NP, M, NS) combined with gene segments from a human seasonal H3N2 influenza virus circulating in 1995 (PB1, HA, NA). The isolates from Minnesota, Iowa and Texas were triple reassortants containing gene segments from the classical swine virus (NP, M, NS,) and the same human virus (PB1, HA, NA) in combination with an avian virus (PB2, PA) (Zhou et al. 1999). By the end of 1999, viruses antigenically and genetically related to the triple reassortant lineage were widespread in the U.S. swine population (Webby et al. 2000) whereas the double reassortant virus did not become established. Interestingly, the double and triple reassortant H3N2 viruses were shown to possess a similar HA encoding gene with identical residues in critical receptor binding regions, suggesting that their different successes were due to factors not associated with the HA and receptor binding pocket. The major difference between the two viruses was the acquisition of two avian polymerase genes (PB2 and PA) in the triple reassortant virus. The human lineage PB1, avian lineage PB2 and PA and swine lineage NP, M and NS found in contemporary swine influenza viruses are referred to as the triple reassortant internal gene (TRIG) constellation (Vincent et al. 2008). Genetic and antigenic evaluation of H3N2 swine influenza isolates since 1998 (Richt et al. 2003; Webby et al. 2004) indicate at least three introductions of human H3 subtype viruses became established in swine, leading to phylogenetic clusters I, II and III. The cluster III viruses have become dominant in North America (Gramer et al. 2007) and have continued to evolve into cluster III variants, also known as cluster IV (Olsen et al. 2006).

The H3N2 viruses not only evolved and became endemic in pigs but also reassorted with extant cH1N1 swine influenza viruses. The vast majority of the resulting reassortant and drift variant viruses since 1998 contain the TRIG. The H1N1 viruses containing the HA and NA from the cH1N1 virus and the TRIG from triple reassortant H3N2 viruses are referred as reassortant H1N1 (rH1N1) and the viruses containing the HA from the classical swine virus and the NA and TRIG from the triple reassortant H3N2 virus are H1N2 viruses (Karasin et al. 2002; Webby et al. 2004) (Fig. 1). Reassortant viruses have become endemic and co-circulate in most major swine producing regions of the U.S. and Canada,

including further drift variants of H3N2 (Webby et al. 2000,2004; Richt et al. 2003; Olsen et al. 2006), H1N2 (Choi et al. 2002; Karasin et al. 2002), and rH1N1 (Webby et al. 2004). H3N1 viruses have occasionally been identified in limited outbreaks but do not appear to circulate widely (Lekcharoensuk et al. 2006; Ma et al. 2006). Moreover, the TRIG was shown to have accepted an avian lineage H2 and N3, producing a novel triple reassortant swine H2N3 in 2006 (Ma et al. 2007). More recently, introduction of H1 viruses with the HA gene of human H1N2 seasonal influenza virus origin (hu-like H1) that are genetically and antigenically distinct from the classical swine H1 lineage were reported in pigs in Canada (Karasin et al. 2006) (Fig. 1). Since 2005, hu-like H1N1 and H1N2 viruses containing the TRIG have emerged in swine herds across the U.S. (Vincent et al. 2009b) that have HA and NA segments most similar to H1N1 and H1N2 human seasonal influenza virus lineages from around 2003.

2.3 Evolution of the H1 Subtype

The well characterized contemporary swine influenza reassortant viruses possessing the ability to spread and become established in U.S. and Canadian swine populations have contained similar TRIG constellations. This would suggest that the TRIG constellation can accept multiple HA and NA types and may confer a selective advantage to viruses possessing this gene cassette (Bastien et al. 2010; Vijaykrishna et al. 2010). Moreover, since the acquisition of TRIG, an increase in the rate of mutation in North American swine influenza isolates appears to have occurred in H1 subtype hemagglutinins. Genetic mutation may be related to antigenic changes if mutations occur in antigenic sites of the HA, potentially resulting in escape from herd immunity. This scenario is in stark contrast with that observed with the cH1N1 viruses prior to acquiring TRIG. Indeed, cH1N1 viruses remained relatively stable genetically and antigenically for at least seven decades (Sheerar et al. 1989; Luoh et al. 1992; Noble et al. 1993; Olsen et al. 1993).

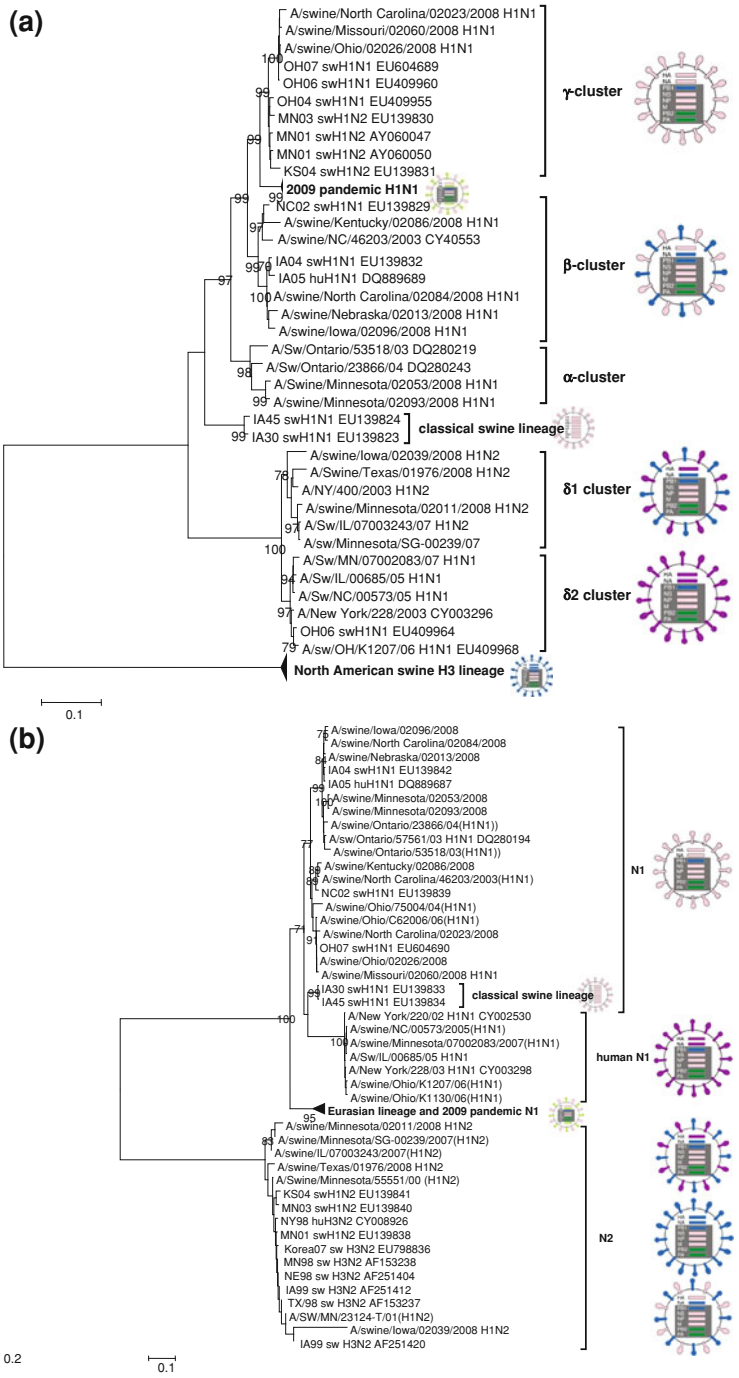
For best representing the evolution of the currently circulating H1 viruses, a cluster classification has been proposed (Fig. 2a). Viruses from the classical H1N1 lineage-HA acquired from the TRIG cassette evolved to form α-, β-, and γ-clusters based on the genetic makeup of the HA gene; whereas H1 subtypes strains with HA genes most similar to human seasonal H1 viruses form the δ-cluster (Vincent et al. 2009b). All four HA gene cluster types can be found with NA genes of either the N1 or N2 subtype. In order to study the evolution and the antigenic relationships among the H1 swine influenza virus subtypes, we recently analyzed 12 different strains, selected from the University of Minnesota Veterinary Diagnostic Laboratory (UMVDL) diagnostic case database (Lorusso et al. 2011). The viruses were isolated from outbreaks of respiratory disease in pigs from diagnostic cases submitted to the UMVDL in 2008 and are representative of each of the postulated four H1 clusters. All gene segments were sequenced and analyzed, and antigenic changes were measured for all twelve viruses using the hemagglutination inhibition (HI) assay

and mapped by antigenic cartography. All 2008 H1 viruses contained the North American TRIG. Furthermore, variation was demonstrated in the six genes that make up the TRIG, but no HA cluster-specific patterns were detected among the genes composing the TRIG constellation. In contrast, an HA cluster-specific pattern was observed for the NA gene. The N1 gene of the α, β and γ cluster of the 2008 H1 viruses and of sequences publicly available each formed a separate clade within the North American N1 cluster. We speculate that the evolution of the H1 gene drives that of the N1 gene as well. Indeed, antigenic drifts that characterize the evolutionary history of the antigenic and phylogenetic clusters of H1 influenza virus in U.S. swine isolates were accompanied by changes in the N1 genes, thus allowing a parallel sub-cluster classification (Fig. 2b). A proper HA/NA pairing in association with the TRIG could optimize viral transmission and replication as shown by recent experiments in pigs. Indeed, experimental coinfection in the lower respiratory tract of inoculated pigs with two phylogenetically and genetically distant viruses, a triple reassortant H3N2 and cH1N1, resulted in the genesis, of all possible HA/NA combinations but only the parental H3N2 was found in two consecutive direct contact pig groups (Ma et al. 2010). These results confirm that multiple reassortments can occur but not all reassortants are readily transmissible.

The viruses representing the classical swine H1 lineage, phylogenetic clusters α, β and γ, had moderate to strong cross-reactivity within a cluster, especially within recent β- and γ-cluster viruses. However, cross-reactivity between clusters was more variable, ranging from no cross-reactivity to strong cross-reactivity, such as between α- and β-cluster viruses. This study suggested that the H1 is evolving by drift while maintaining the TRIG backbone, and that the resulting viruses differ genetically and antigenically with obvious consequences for vaccine and diagnostic test development. In 2008 and 2009, α cluster H1 viruses were rarely isolated from influenza outbreaks in pigs in North America, and while β cluster H1 viruses are still common, they occur with less frequency than the more dominant subtypes from the the γ and δ viruses. Since the acquisition of TRIG, the H1 of the classical swine lineage, under apparent evolutionary pressure, has developed multiple amino acid changes in the putative antigenic sites. The γ viruses are chronologically the newest H1 variants and it cannot be ruled out that the same mechanisms will be responsible for further H1 cluster variants. The genetic diversity within the H1 clusters was confirmed functionally by the demonstrated loss in cross-reactivity in the HI assay between H1 clusters overall. It is likely that, as a consequence of evolutionary and immunogical pressures, the H1 will continue to mutate in the future, allowing evasion of the immune system of the host or only partially protective immunity.

2.4 Human-Like H1 Viruses

Since 2005, H1N1 and H1N2 viruses with the HA gene derived from human viruses have spread across the U.S. in swine herds forming the δ-cluster H1 (Vincent et al. 2009b) (Fig. 1). The HAs from the human-like (hu) swine H1



◀**Fig. 2** Neighbor-joining trees inferred from multiple nucleotide sequence alignment of segment 4 (HA, **a**) and segment 6 (NA, 2**b**). **a** shows four H1 clusters of viruses, H1α, H1β, H1γ and H1δ (human-like H1) as indicated by the bars on the right of the tree. In both trees, the HA cluster specificity is indicated. The genomic constellation of each clade is indicated by the images on the right side of the tree. Classical swine lineage is color coded pink, avian lineage is coded green, human lineage is coded blue or purple. Light green indicates the Eurasian avian/swine lineage. Classical swine lineage-HA gene (**a**) was acquired by the TRIG cassette and evolved overtime to form α-, β- and γ- clusters. The introduction of human seasonal HA from H1N2 and H1N1 gave rise to δ cluster viruses differentiated phylogenetically by two distinct sub-clusters, δ1 and δ2 (**a**). Similar to the δ-cluster viruses in the HA phylogenetic analysis, β-viruses have split into two sub-clusters (**b**). Phylogenetic analyzes were conducted in MEGA4. Statistical support was provided by bootstrapping over 1,000 replicates and bootstrap values >70 are indicated at the correspondent node. The scale bars indicate the estimated numbers of nucleotide substitutions per site. human (*Hu*), swine (*Sw*)

viruses are genetically and antigenically distinct from classical swine lineage and derivatives. Indeed the putative antigenic site in the HA1 of the hu-like viruses possesses typical human lineage residues in contrast to that found in the HA1 of the α-, β- and γ-clusters (Lorusso et al. 2011). However, their TRIG genes are similar to those found in the TRIG cassette of the contemporary swine triple reassortant viruses (Vincent et al. 2009b). The HA from the δ-cluster viruses were shown to have most likely emerged from at least two separate introductions of human seasonal HA from H1N2 and H1N1 viruses being differentiated phylogenetically by two distinct sub-clusters, δ1 and δ2, respectively, (Lorusso et al. 2011; Vincent et al. 2009a). Viruses belonging to the δ-cluster were shown to be paired either with a N1 or N2 gene consistently of human lineage and not of swine lineage N1. δ1-subcluster viruses, first detected in 2003, showed an N2 gene preference whereas δ2-subcluster viruses, first detected in 2005, showed an N1 preference (Fig. 2b) initially but have subsequently begun to reassort. Limited HI cross-reactivity was demonstrated between the δ1 and δ2 viruses thus supporting the scenario assumed by the phylogenetic analysis (Fig. 2a). The hu-H1 viruses have become one of the major subtypes of influenza virus isolated and characterized from swine respiratory disease outbreaks. Indeed, if we consider the time period 2008–2010, the incidence of hu-H1 in swine respiratory disease outbreaks has dramatically increased. In 2008, 85% of the influenza viruses isolated from swine diagnostic cases submitted to the UMVDL were shown to be of the H1 subtype. Most of the H1 isolates (up to 78%) were of the γ- and β-cluster with the γ-cluster viruses found in slightly higher numbers, whereas δ-cluster viruses represented approximately 20% of the total. However, in 2009 the epidemiologic scenario changed. While the influenza A viruses isolated were mostly H1 subtype (five-fold more than the H3 subtype), the number of δ viruses now represented 40% of the total, thus quickly becoming the dominant subtype isolated from cases of respiratory disease. β- and γ-cluster viruses were 35 and 23% of the total H1 clusters represented, respectively. The same trend was shown in the early months of 2010 as well, with a slight increase in the number of δ-cluster viruses compared to the γ-cluster viruses, cluster IV H3 subtype viruses, and the newly emerged

2009 pandemic H1N1. An experimental in vivo study in 4-week-old pigs with an H1N1 isolate of the δ2-subcluster demonstrated differences in kinetics of lung lesion development, viral load in the lung and nasal shedding when compared to a virulent rH1N1 in the β-cluster. This study suggested the emerging virus genotype may not have been fully adapted to the swine host since virus replication in the lung and virus shedding from the nose were reduced compared to a contemporary rH1N1 (Vincent et al. 2009b). A more recent pathogenesis and transmission study in pigs comparing viruses in the δ1- and δ2-subclusters recapitulated the phenotypic differences seen in the initial study; however, the δ1-subcluster virus studied demonstrated increased virulence and nasal shedding over the δ2-subcluster viruses (Ciacci-Zanella, unpublished). Further studies are warranted in order to monitor the evolution of δ-cluster viruses. The presence of typical "human-like" residues in the receptor binding pocket in the HA of two of the δ-cluster viruses isolated in 2008 demonstrates that although these viruses have replicated in pigs for over five years, the swine viruses may preserve human-adapted receptor binding phenotypes (Lorusso et al. 2011). This preservation of human-like residues in the swine host may allow potential novel reassortant influenza viruses, including the δ-cluster swine viruses, to spill back into the human population. Escaping the immune response by changing the external makeup is a well-known strategy that influenza viruses adopt. The acquisition of human HA segments by the TRIG cassette platform were shown to be entirely different from those of the classical swine lineage and further drift derivatives provided an important antigenic advantage for these reassortant viruses. Indeed, the number of influenza outbreaks in which δ-cluster viruses were recognized as causative agents increased in the recent years. Moreover, geographical regions have differing cluster variants circulating, thus further complicating vaccine strain selection.

2.5 2009 Pandemic H1N1 in Pigs

In the early spring of 2009, the United States, Canada and Mexico reported community outbreaks of pneumonia in humans caused by a novel H1N1 influenza A virus. This virus subsequently spread across the globe at a high rate, prompting the WHO to declare a pandemic in June 2009 (Garten et al. 2009). Retrospectively, the earliest known case was identified February 24, 2009, in a baby from San Louis Potosi, Mexico (http://news.sciencemag.org/scienceinsider/2009/07/yet-another-new.html). This novel pandemic H1N1 possesses a unique genome with six gene segments (PB2, PB1, PA, HA, NP and NS) most closely related to the triple reassortant influenza viruses of the North American swine lineage, and the M and NA genes derived from a Eurasian lineage of swine influenza viruses (Dawood et al. 2009). The 2009 pandemic influenza became infamously known as "swine flu" due to the phylogenetic origin of the gene segments. However, since the recognition of the outbreak, infection in humans has not been connected to pig exposure (Dawood et al. 2009). Indeed, as it was believed to have occurred in 1918

(Webster 2002), humans transmitted the novel virus to pigs, as most of the initial documented swine outbreaks were preceded by reported human influenza-like illness during the human pandemic (Cohen 2010). The 2009 pandemic H1N1 was promptly shown to replicate efficiently in the lower and upper respiratory tract of infected pigs and to cause a clinical disease comparable to that typically observed during common, enzootic influenza virus infection in swine (Lange et al. 2009; Vincent et al. 2009a). Early reference to the 2009 pandemic H1N1 as "swine flu" led to unnecessary alarm over the safety of pork meat products and culminated in the ban of exported pork from the U.S. by several countries, resulting in billions of dollars in lost revenue for the swine industry (http://agriculture.house.gov/testimony/111/h102209/Butler.pdf). However, contamination of fresh pork meat with the novel virus was experimentally excluded (Vincent et al. 2009a). Immediately after the onset in humans, cases of infection of pigs with the pandemic 2009 H1N1 were reported in different areas of the world (http://www.oie.int/wahis/public.php?page=weekly_report_index&admin=0). The first case was detected on April 28, 2009 in Canada (Leslieville, Alberta) in a farm with pigs that were not previously vaccinated against swine influenza (Howden et al. 2009; Weingartl et al. 2010). The source of the outbreak was linked to a worker who showed symptoms of influenza-like disease (Howden et al. 2009). Pigs infected with the 2009 pandemic H1N1 were first detected in the U.S. in a farm in Indiana in November 2009 (Lowe et al. 2010). Based on recent data the 2009 H1N1 continued to spread from humans to susceptible pigs with subsequent sustained pig-to-pig transmission and, thus establishing yet another endemic virus in swine populations. Importantly, none of the eight genes of the 2009 pandemic H1N1 cluster tightly with the genes of swine influenza viruses circulating in the U.S. prior of the outbreak in humans (Lorusso et al. 2011; Smith et al. 2009). In the phylogenetic analyzes of each gene segment, the 2009 pandemic H1N1 formed a distinct and independent branch from the U.S. swine lineage genes of the 2008 H1 isolates evaluated as well as swine virus sequences available from GenBank. This suggests that neither the 2009 pandemic H1N1 nor closely related progenitor viral genes were present in U.S. swine influenza viruses prior to 2009 (Fig. 2a, Lorusso et al. 2011). A closely related progenitor virus with the same 8-gene constellation has yet to be identified in swine or other species, although a 2004 swine virus with 7/8 of the 2009 pandemic H1N1 genome was identified in Hong Kong, China (Smith et al. 2009).

The HA of the 2009 pandemic H1N1 is most closely related to the North American swine γ-cluster H1 lineage viruses (Fig. 2a). Limited serologic cross-reactivity was demonstrated in HI tests using two 2009 pandemic H1N1 human strains as antigens against sera from pigs immunized with 2007–2008 (notably γ-cluster) swine viruses (Vincent et al. 2010). Thus, prior exposure to some H1 subtypes is likely to provide swine with some level of protection against infection with the 2009 human pandemic H1N1. This is also suggested by data from human epidemiological studies that showed high prevalence of neutralizing antibodies against 2009 pandemic H1N1 in people born before 1930 (Itoh et al. 2009; Munster et al. 2009). Moreover, immunization in mice with human H1N1 viruses

that circulated before 1945 (e.g. specific antibodies against 1918 H1N1 or related viruses) is sufficient for immune protection from the 2009 pandemic H1N1 (Manicassamy et al. 2010). Furthermore, 2009 pandemic H1N1 viruses cause sustained human-to-human transmission and there are several case reports and experimental studies demonstrating human-to-swine (Howden et al. 2009), swine-to-swine (Vincent unpublished; Lange et al. 2009; Brookes et al. 2010) and swine-to-human transmission (Weingartl et al. 2010). Thus, the 2009 pandemic H1N1, being a virus shared between people and pigs, has the potential to further change the epidemiology of influenza viruses in human and swine populations.

2.6 Exceptional Influenza A Subtypes: H2N3 and H4N6

Two H2N3 influenza viruses were isolated in 2006 from clinically affected pigs from two different farms in the central U.S. (Ma et al. 2007). The epidemiologic link identified between the two farms, besides the geographical location, was the use of water collected from open-air ponds for pig drinking water and cleaning the barns. This subtype was not reported in pigs before and has not been identified since. Sequencing demonstrated both viruses were H2N3 influenza A viruses sharing 99.3–99.9% genetic homology. Although the H2N3 viruses contained the typical North American TRIG, the HA segment was similar to an avian influenza virus H2N3 isolated from mallards and the NA sequence was similar to an avian influenza virus H4N3 isolated from blue-winged teal. Importantly, the predicted HA1 protein displayed an amino acid constellation in the receptor binding area suggesting a preferential affinity to the mammalian receptor. This HA mutation resembled that of the initial reassortant human influenza isolates found in the beginning of the 1957 H2N2 pandemic. To investigate the transmission and pathogenesis features of the novel virus, in vivo studies with pigs, mice and ferrets were conducted. Both swine and ferrets were shown to efficiently transmit virus to contact animals; moreover the virus was lethal to young mice. The combined data demonstrating the ability of the H2N3 viruses with a typical avian-origin HA to replicate in three different animal models confirmed adaptation to the mammalian host, indicating a potential risk to the human population. However, serological studies conducted on workers exposed to H2N3 infected pigs showed no evidence of zoonotic transmission (Beaudoin et al. 2010).

A similar scenario was described in October 1999 in a swine farm in Canada. This farm was located near a lake on which large numbers of waterfowl congregate each fall and from which the farm drew water. The source of infection was postulated to have come from ducks on the adjacent lake. Viruses isolated from infected pigs, were shown genetically and antigenically to be wholly avian H4N6 viruses of the North American avian lineage (Karasin et al. 2000). Moreover, the wholly avian H4N6 virus spread from pig-to-pig (Olsen 2002), but has not been detected outside the primary swine farm system. Interestingly, even in this case of a wholly avian virus, the receptor binding pocket of the putative HA protein

displayed residues postulated to be associated with mammalian receptor binding (Karasin et al. 2000).

2.7 Triple Reassortant Swine Viruses Found Outside North America

China is a major area of swine and poultry production. Influenza virus infection in pigs was first described in 1918 in China coincident, as in other areas of the world, with the Spanish flu pandemic in humans (Brown 2000). It has been documented that four subtypes (H1N1, H1N2, H3N1, and H3N2) are circulating in Chinese pigs (Shu et al. 1994; Guan et al. 1996; Peiris et al. 2001; Xu et al. 2004; Qi and Lu 2006; Qi et al. 2009). Studies from 1976 to 1982 revealed cocirculation of classical swine H1N1 and human-like H3N2 and double reassortant H3N2 viruses containing the surface genes HA and NA from the human viruses and the internal genes from swine H1N1 viruses (Shortridge et al. 1979, 1987; Xu et al. 2004). In 1993–1994, avian-like H1N1 viruses were detected in pigs in southern China (Guan et al. 1996). In 2004, a reassortant H1N2 virus containing the NA gene of a human H3N2 and the remaining seven genes of the classical H1N1 swine viruses emerged (Qi and Lu 2006). Recently, interspecies transmission of human H1N1, avian H5N1 and avian H9N2 to pigs has been reported (Peiris et al. 2001; Yu et al. 2007; Zhu et al. 2008). Moreover, recent studies revealed the presence of novel avian-like H1N1strains that seem to be derived from the European swine H1N1 viruses and novel triple reassortant H1N2 carrying the TRIG. These novel triple reassortant viruses have the HA of the classical swine lineage whereas the NA is of human origin (Yu et al. 2009). The epidemiology of influenza viruses in the Chinese swine population is complex and worthy of increased monitoring and reporting.

Currently, at least four different subtypes of swine influenza viruses, classical H1N1, H1N2, H3N1 and H3N2, are circulating in South Korean swine herds with H1N2 being the dominant subtype (Jung and Chae 2005; Shin et al. 2006; Jung et al. 2007; Song et al. 2007). Multiple HA lineages are present for the H1 and H3 subtypes (Pascua et al. 2008). H1N1 viruses currently circulating show the HA is closely related to that of the first H1N2 isolated in South Korea in 2002, A/swine/Korea/CY02/02. However, more recent H1N2 isolates contained HA genes closely related to an H1N2 isolate (A/swine/MN/16419/01) reported in USA. These findings suggest that the HA genes from recent H1N2 viruses originated from viral sources other than the pre-existing H1N2 isolates in Korea, such as those from USA. The phylogeny of the H3 subtype is more complicated. A recent study showed that four different H3 lineages cocirculate in Korean swine. The first lineage displayed an H3 identical to that described in USA in the earliest H3N2 isolates containing the TRIG constellation, A/swine/TX/4199/2/98 (cluster I), whereas the HA gene of the second lineage appeared to be derived from the American cluster III H3N2. Another strain was found to possess an H3 similar to

those of the American cluster II H3N2. The remaining lineage contains new emerging reassortant viruses in which the HA gene originated from an earlier human-like isolate (A/NY/647/95). All the viruses characterized from the period 2002–2007 in South Korea were shown to possess the TRIG. Most recently, two H5N2 viruses with the surface glycoproteins from the Eurasian avian lineage were isolated from swine. While the first of the two was a wholly avian virus, the second was a double reassortant between an avian virus (PB1, HA, NA, NS) and a triple reassortant swine H3N1 (PB2, PA, NP, M) (Lee et al. 2009).

The TRIG genome constellation of influenza viruses from Canadian and American pigs has not been described in Europe, whereas recent studies reveal its presence in China and Korea. Many major swine producing regions of the world are under-represented in the influenza genome databases and reports in the literature documenting the status of influenza in swine are likewise limited or absent. However, the global spread of the 2009 pandemic H1N1 carrying a modified TRIG constellation represents a new viral source for the TRIG with an unpredictable consequence to the epidemiology of swine influenza viruses worldwide. Thus, increased and sustainable global surveillance for influenza viruses in swine is needed in order to develop control and prevention programs for swine health as well as rapid detection and reporting of emerging zoonotic influenza threats.

3 Conclusions

Currently, up to seven antigenic HA clusters and subtypes are cocirculating in pigs in Canada and the United States: α-, β-, γ-, $\delta 1$- and $\delta 2$-cluster viruses of the H1 subtype, 2009 pandemic H1N1 and the cluster IV triple reassortant H3N2. The HA of α-, β- or γ-cluster H1 viruses can be paired with either N1 or N2 genes. The N2 was introduced from humans in the 1990s with the H3N2 and is well-established in the swine population. However, $\delta 1$- and $\delta 2$-subcluster human-like viruses where shown to be preferentially paired with an N1 or N2 genes consistently of human lineage. $\delta 1$-subcluster viruses showed an N2 gene preference whereas $\delta 2$-subcluster viruses showed an N1 preference.

The key element in understanding influenza virus epidemiology in all of the influenza A viruses resides in the fascinating plastic nature of their genome. Whereas pandemic influenza outbreaks occur following rare antigenic shifts, established influenza viruses perpetually undergo antigenic drift allowing survival in the host population. The 2009 pandemic H1N1 underscores the potential risk to the human population from additional influenza virus subtypes and genotypes with the swine influenza TRIG backbone and demonstrates the potential for viruses with genes from swine lineages to emerge and cause illness in the human population. On the other hand, from the swine industry point of view, pigs have been the victim of human-adapted seasonal influenza viruses, as well as the 2009 pandemic H1N1. Although the 2009 pandemic H1N1 does not pose a greater risk in terms of clinical disease over viruses endemic prior to 2009, its emergence in

the swine populations makes influenza control strategies exceedingly difficult. Additionally, there is a concern for the novel pandemic H1N1 virus to pick up or contribute genes of different sources in the swine host, potentially generating further reassortant viruses with potential implications for the human and swine populations. Indeed, a novel reassortant virus has been recently found in pigs in Hong Kong. It was shown to have the NA gene of 2009 pandemic H1N1 origin, the HA gene of the avian-like H1N1 swine isolates, whereas the remaining genes are TRIG-derived (Vijaykrishna et al. 2010). This is the first documented event of reassortment involving the 2009 pandemic H1N1 but not likely the last.

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History and Epidemiology of Swine Influenza in Europe

Ian H. Brown

Abstract In Europe, swine influenza is considered one of the most important primary pathogens of swine respiratory disease and infection is primarily with H1N1, H1N2 and H3N2 influenza A viruses. The antigenetic characteristics of these viruses distinguish them from others circulating at a global level in pigs. These viruses have remained endemic in European pig populations but significant differences in the circulation of these strains occur at a regional level across Europe. The dynamic of co-circulation of viruses, impact of prior immunity, husbandry practices and other local factors all contribute to the complex epidemiology. Surveillance programmes in European pigs did not reveal the presence of pandemic H1N1 virus prior to its detection in humans in 2009 but there is evidence that the virus can be maintained in European pigs even when there are relatively good levels of herd immunity to other H1 viruses. Evidence for the pig as a 'mixing vessel' of influenza viruses of non-swine-origin has been demonstrated in Europe on several occasions. Furthermore significant and highly variable genetic diversity occurs at the whole genome level for all virus subtypes and this has contributed to changing patterns of virus epidemiology over time.

Contents

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Current Topics in Microbiology and Immunology (2013) 370: 133–146
DOI: 10.1007/82_2011_194

Published Online: 11 January 2011

1 Introduction

Swine influenza (SI) is a highly contagious acute viral disease of the respiratory tract in pigs which is distributed worldwide. The disease is economically damaging primarily due to weight loss and reduced weight gain but on occasions infection can be much more severe, particularly if exacerbated by the presence of other pathogens or factors. In the UK the financial loss resulting from reduced weight gain in pigs alone due to SI has been estimated at approximately £7 per pig, equivalent to a total loss of £60 million per annum (Kay et al. 1994). It is well recognised that influenza can be a component with a multifactorial aetiology associated with porcine respiratory disease complex, involving numerous viruses and bacteria (Hansen et al. 2010). In Europe, SI is considered one of the most important primary pathogens of swine respiratory disease. The characteristics of the viruses responsible share some similarities with counterpart strains in North America and Asia but also possess a number of significant differences. The epizootiology of SI in Europe has historically been different from other parts of the world and together with approaches for control remains distinct.

Historically, the epidemiology of influenza virus in pig populations is unique and can clearly be differentiated from related strains in other hosts including humans. There is periodic exchange of virus or virus genes between different hosts through interspecies transmission and/or genetic reassortment. Analysis of all the stable lineages of influenza viruses in pigs has revealed that they originated from an avian or human source, acquired the capability to infect and transmit between individual pigs, thereby persisting within swine populations. Viruses with high fitness acquire the capability to become established and endemic in populations. Clear differences can be determined in lineages of viruses established contemporaneously. Different lineages of viruses can be identified based on spatio-temporal characteristics. It is not uncommon for multiple subtypes of virus to be co-circulating in discreet populations. This gives rise to the potential for further genetic reassortment resulting in the production and emergence of further viruses that are capable of infecting and spreading within pigs. Occasionally, such viruses may also cross the species barrier once more into other host populations (Olsen et al. 2006). The consequences of such events lead to independent evolution of influenza viruses in pigs, especially at continental level when clear differences in the circulating viruses can be determined (Brown 2008).

The so-called classical H1N1 viruses which were first observed in 1918 in the United States and derived from a progenitor strain responsible for the human pandemic at the time, did spread around the globe but only circulated in European pigs for approximately 15 years and were replaced by H1N1 viruses of avian origin that entered European pig populations in the early 1980s (Pensaert et al. 1981). These so-called avian-like H1N1 viruses have become highly adapted and extremely successful at transmitting between, and being maintained within pig populations, frequently in association with significant disease problems. The dynamic is further complicated by frequent transmission of virus from infected humans to pigs and this event is highly relevant in the context of understanding the dynamics and future pathways for the evolution of pandemic influenza A virus of H1N1 subtype that emerged in humans in 2009 [A(H1N1) pdm09] in global pig populations.

2 Epidemiology

Influenza A viruses of subtypes H1N1 and H3N2 have been reported widely in European pigs, associated frequently with clinical disease. These include classical swine H1N1, 'avian-like' H1N1 and 'human-like' H3N2 viruses. These viruses have remained largely endemic in European pig populations and have been responsible for one of the most prevalent respiratory diseases in pigs. Although usually regarded as an endemic disease, epidemics may result when influenza infection occurs in an immunologically naive population (which can be linked to significant antigenic drift) or through exacerbation by a variety of factors such as poor husbandry, secondary bacterial or viral infections and cold weather. Virological surveillance for SI viruses in pigs suffering acute respiratory disease in Belgium, UK, Italy, France and Spain from 2006 to 2008 detected a total of 169 viruses of which 81 were classified as 'avian-like' H1N1, 36 as human-like H3N2 and 47 as swine H1N2. Only five novel reassortant viruses were identified (Kyriakis et al. 2011).

Serosurveillance results in Great Britain (GB) indicated that more than half of adult pigs in the national population had been infected with one or more influenza A viruses during their lifetime, including fourteen percent of pigs which had been infected with influenza viruses of both human and swine origin (Brown et al. 1995b). In another study in Spain, 83% of herds and 76% of animals studied were seropositive for one or more influenza virus subtypes (Maldonado et al. 2006). This provides some indication of the risk of genetic reassortment of influenza A viruses in pigs.

2.1 Regional Variation

Ongoing surveillance programmes in Europe have revealed continuous circulation of H1N1, H3N2 and H1N2 viruses. In Central Europe activity with SIV is generally low with only avian-like H1N1 viruses being established. Levels of

seroprevalence across Europe will vary greatly and may be impacted by production type and system, together with local factors. High seroprevalence has been reported in Belgium, Germany, Italy and Spain with levels often in excess of 50% for most of the subtypes. Generally the more intensive production areas result in a greater diversity of antibody to one or more virus subtypes. In less intensive production areas such as in Ireland, Czech Republic and Poland, seroprevalence was generally lower and more specifically related to avian-like H1N1 with low or no seroprevalence detected to H1N2 and H3N2 (Van Reeth et al. 2008).

Significant differences in circulation of endemic swine strains occur across Europe; broadly, avian-like H1N1 viruses are widely distributed in all countries and H1N2 is becoming increasingly widespread (Kyriakis et al. 2011). However, some differences are noted in the circulation of human-like H3N2 viruses with extensive circulation and often high seroprevalence in herds in Southern Europe but the virus appears to be absent in France and the UK (Kyriakis et al. 2011). The basis for these significant epidemiological differences is not fully understood, however the complex dynamic of co-circulation of viruses, impact of prior immunity, husbandry practices and other local factors will all contribute to the complex epidemiology. Some of the higher density pig breeding areas in Spain experienced very high levels of virus circulation as detected through serological surveys with an excess of 85% of sows and 80% of fatteners seropositive for one or more strains of influenza virus (López-Soria et al. 2010).

3 Virus Strains

3.1 Classical H1N1

This virus became endemic in pigs throughout Europe with a seroprevalence of 20–25% (Zhang et al. 1989; Brown et al. 1995b) but following the emergence of 'avian-like' H1N1 virus the classical H1N1 virus has disappeared (Brown 2000). Throughout the period, these viruses remained relatively stable antigenetically with no evidence of involvement in virus reassortment (Brown et al. 1997).

3.2 'Human-Like' Viruses

Infections in pigs with the prevailing human subtypes also occur under natural conditions. Shope (1938) presented serological evidence that human to pig transmission could occur, but it was not until the isolation of Hong Kong H3N2 virus from pigs in Taiwan in 1970 (Kundin 1970) that investigations began to examine the potential transmission of human strains to pigs. Although no disease was reported among infected pigs, in the next several years H3N2 viruses were

isolated regularly from European pigs (Tumova et al. 1976; Ottis et al. 1982) and/or antibody was demonstrated (Harkness et al. 1972; Tumova et al. 1976) in European swine populations. Since 1984, these viruses have been associated with outbreaks of clinical influenza in pigs throughout Europe (Aymard et al. 1985; Haesebrouck et al. 1985; Pritchard et al. 1987) with infections frequently characterised by high seroprevalence (Tumova et al. 1980; López-Soria et al. 2010). In Europe, contemporary influenza viruses of H3N2 subtype are antigenically, related closely, to early human strains such as A/Port Chalmers/1/73. The limited immune selection in pigs facilitates the persistence of these viruses, which may in future transmit to a susceptible human population. However, some viruses although related closely to the prototype human viruses have antigenic differences in the surface glycoproteins and may cocirculate with the former strains (Haesebrouck and Pensaert 1988; Brown et al. 1995a). Recently, there has been considerable antigenic variation in the HA gene of 'human-like' H3N2 viruses due to marked genetic drift and this has led to an apparent increase in epizootics attributable to this virus (DeJong et al. 1999). These recent viruses appear only distantly related antigenically to the early prototype strains.

Human H1N1 viruses (see also Sect. 3.5) can also infect pigs, but although pig to pig transmission has been demonstrated under experimental conditions most strains are not readily transmitted among pigs in the field. Although there is serological evidence that they are present in European pigs (Brown et al. 1995b), it is most likely that they only occur through frequent transmissions of the prevailing strains from humans and are not apparently maintained in pigs independently of the human population although individual genes may be following genetic reassortment (Brown 2000).

3.3 'Avian-Like' H1N1 Viruses

Since 1979 the dominant H1N1 viruses in European pigs have been 'avian-like' H1N1 viruses which are antigenetically distinguishable from classical swine H1N1 influenza viruses, but related closely to H1N1 viruses isolated from ducks (Pensaert et al. 1981; Scholtissek et al. 1983). These 'avian-like' viruses appear to have a selective advantage over classical swine H1N1 viruses which are related antigenically, since in Europe they have replaced classical SI virus (Campitelli et al. 1997; Brown 2000). Within 2 years of the introduction of 'avian-like' viruses into pigs in GB, classical swine H1N1 apparently disappeared as a clinical entity. In Europe, avian H1N1 viruses were transmitted to pigs, became established, and have subsequently been reintroduced to turkeys from pigs causing economic losses (Ludwig et al. 1994; Wood et al. 1997). These viruses remain the dominant strain in pigs in many European countries.

3.4 H1N2 Viruses

Influenza A H1N2 viruses, derived from classical swine H1N1 and 'human-like' swine H3N2 viruses were isolated in France in the late 1980s (Gourreau et al. 1994). These viruses inherited the HA gene from classical swine H1N1 and the NA gene from the swine adapted human virus, however, although they were associated with clinical disease they did not appear to spread widely. In 1994 H1N2 influenza viruses related antigenically to human and 'human-like' swine viruses emerged and became endemic in pigs in GB (Brown et al. 1995a) frequently in association with respiratory disease. Subsequently these viruses spread to pigs in the rest of Europe (Van Reeth et al. 2000; Marozin et al. 2002) and became endemic. Occasionally viruses of H1N2 subtype carrying other genes notably HA derived from other swine strains are detected but lack the ability to persist and become truly established in pig populations. In addition, genetic diversity of the H1N2 virus has further been reported in strains co-circulating in Germany with different combinations of neuraminidase (Zell et al. 2008). Virological surveillance for SI viruses in pigs in five European countries between 2006 and 2008 revealed only five (2.9%) novel reassortant viruses out of a total of 169 viruses identified: two H1N1 viruses had a human-like HA and three H1N2 viruses had an avian-like HA (Kyriakis et al. 2011).

3.5 A (H1N1) pdm09

In March 2009, retrospective analysis revealed that the first cases of human infection with a novel H1N1 virus showing close similarity to swine strains detected in North America was first reported in people in Central America. Whether this is where the pandemic truly began or whether the virus was moved to Mexico through infected humans or animals still remains uncertain. Nevertheless, detailed studies on the genetic characteristics of the virus revealed that it contained a unique gene constellation not previously described or reported in viruses in humans or pig populations (Garten et al. 2009). The virus contained two gene segments derived from avian strains of North American lineage, one gene from human influenza, three genes from classical swine viruses (still circulating in North American and Asian pigs) and significantly, two segments from a Eurasian swine lineage (NA and MP). Detailed analysis has revealed that there is a gap in the ancestry of these viruses providing some level of uncertainty as to the precise origin. However, the closest match and similarity to viruses circulating in pigs in at least seven of eight of the genes segments places the origin of the pandemic virus in pigs as highly probable (Smith et al. 2009). Contemporary surveillance programmes in European pigs have not revealed the presence of this virus prior to its detection in the human population (Kyriakis et al. 2011).

Given the high similarity in all of the gene segments to potential progenitor strains circulating in pigs, it would appear inevitable that the virus would have a high capability to spread from humans to pigs following subsequent global dispersion of the virus through the human population. Shortly after the rapid spread of A (H1N1) pdm09 virus in the human population, infection in pigs was reported on a global scale. The first documented cases in European pigs occurred in Northern Ireland (United Kingdom) in September 2009 in association with mild respiratory disease in fattening pigs (Welsh et al. 2010). This study also reported infection in asymptomatically infected pigs, providing further evidence for apparent subclinical infection which may be related to other factors such as immune status or the virus itself (Welsh et al. 2010). This was soon followed by cases in 22 OIE member countries from five continents reported under the new and emerging diseases category to the OIE in the period up to September 2010 (OIE 2010). Furthermore, a number of studies revealed that pigs are highly susceptible to infection and present with typical clinical signs as seen with other strains of influenza (Brookes et al. 2010; Lange et al. 2009). The virus readily transmits between susceptible animals and therefore it would be postulated that the virus could become very readily established in pig populations following primary contact with infected humans. The number of cases detected in Europe and elsewhere in the world to date reveal the likely establishment of the virus through pig to pig transmission rather than reliance on exposure to infected humans in all cases. Understanding the dynamic and interplay between infection of humans and pigs is complex, especially compounded by the close genetic similarity in the viruses even though they may be circulating independently in both human and swine populations. Since it is well established that influenza viruses evolve independently in host populations over time as demonstrated through the establishment of H3N2 viruses in both humans and pigs since 1968 (Olsen et al. 2006), it can be expected that some divergence in the evolution of the A(H1N1) pdm09 virus will occur in the future since it will be subjected to different evolutionary pressures in different host populations (Dunham et al. 2009). At present, sufficient divergence has not occurred in the virus to enable studies to reveal with high degree of certainty the direction of transmission between pigs and humans following widespread infection in the human population.

The future dynamics of infection in European pigs will be complex, especially taking into account the immune status and characteristics of virus circulating in pig populations. The impact of prior immunity to other ‘endemic’ H1N1 viruses upon infection with A(H1N1) pdm09 is not yet fully understood at field level but it would be expected, based on previous data with swine H1 viruses that some protection may be afforded against incursion of the pandemic strain. Nevertheless where surveillance programmes remain robust there is evidence that the A (H1N1) pdm09 virus can be maintained in pigs even when there are relatively good levels of herd immunity to other H1 viruses (Brown IH, personal communication). Following the widespread emergence of avian-like H1N1 in European pigs, classical H1N1 viruses disappeared, presumably because they lacked fitness compared to the highly efficient and successful avian-like swine lineage viruses.

3.6 Novel Reassortant Viruses

Continued co-circulation of influenza A viruses in pigs can result in the production of new reassortant viruses. Many herds are infected endemically, often with more than one subtype and this provides a moderate risk of genetic reassortment. This is an ongoing process with frequent genetic exchange between co-circulating variants of the same virus. Rarely, genetic reassortment occurs between viruses of different subtypes and leads to the production of viable virus that either has a modified genotype or a 'new' phenotype.

Evidence for the pig as a mixing vessel of influenza viruses of non-swine-origin has been demonstrated in Europe by Castrucci et al. (1993), who detected reassortment of human and avian viruses in Italian pigs. Phylogenetic analyses of human H3N2 viruses circulating in Italian pigs revealed that genetic reassortment had been occurring between avian and 'human-like' viruses since 1983 (Castrucci et al. 1993). All of these viruses retained an H3N2 serotype but inherited avian (replacing human) virus genes which encode the 'internal' proteins of the virus.

Further evidence for the emergence of new strains which are able to spread widely in pigs following genetic reassortment was the appearance of H1N2 virus in GB in 1994 (Brown et al. 1995a) before apparent spread to the rest of Europe. The H1N2 viruses derived from a multiple reassortant event over a number of years involving human H1N1, 'human-like' swine H3N2 and 'avian-like swine' H1N1 (Brown et al. 1998). These viruses are genetically and antigenically very distinct from viruses of the same serotype that had been present in pigs in France briefly in the late 1980s.

Significant and highly variable genetic diversity occurs at the whole genome level within all of the virus subtypes that occur naturally in pigs in Europe. This variability is most easily identified for both H1N2 and H3N2 subtypes. Reassortment with avian-like swine H1 and human-like swine H3N2 viruses acquiring HA and NA, respectively, with avian-like swine genes encoded internal proteins has been reported on numerous occasions. This can include the acquisition of the neuraminidase gene from different progenitor strains that have been maintained within pigs (Balint et al. 2009).

The continuous co-circulation of viruses in European pigs presents significant opportunity for continual genetic mixing and from time to time, novel virus subtypes or genotypes are detected. The appearance of new serotypes in pigs does not always result in them becoming established in an immunologically naïve population. The long term persistence of these reassorted viruses is usually short with such strains failing to establish stable genetic lineages within European pigs. The occurrence of such novel subtypes has been reported on a number of occasions and includes H3N1 in pigs in Italy (Moreno et al. 2009) and the UK (Brown 2000) but these viruses apparently fail to persist. An H1N7 virus isolated from pigs in GB in 1992 was antigenically unique being derived from human and equine viruses (Brown et al. 1994) but apparently failed to spread within pigs or to other

species. However, the emergence of new viruses in pigs, even if they fail to become established may have implications for other hosts such as humans.

Following the emergence of A(H1N1) pdm09, opportunity for reassortants through co-infection of herds has already been reported. In the UK an H1N2 virus has acquired the internal protein gene constellation of the A(H1N1) pdm09 virus with the external glycoproteins HA and NA derived from contemporary swine endemic H1N2 viruses (Howard et al. 2011). In addition, an H1N2 virus has been reported in Italy in which the NA only of the A(H1N1) pdm09 virus has been replaced by the N2 gene from either H1N2 or H3N2 European swine viruses (Moreno et al. 2011).

4 Evolutionary Dynamics

Following transmission of an influenza virus to pigs from birds or humans the virus needs to adapt to the new host before becoming pathogenic for pigs. The available evidence suggests that this process can take many years after the initial introduction of virus. The newly introduced/emerged influenza virus may undergo many pig to pig transmissions because of the continual availability of susceptible pigs. The mechanisms whereby an avian virus is able to establish a new lineage in pigs are not fully understood although following the introduction of an avian virus into European pigs in 1979 the virus was relatively unstable genetically for approximately 10 years (Ludwig et al. 1995). The continual genetic exchange between influenza viruses is likely to result in the emergence of 'genetic variants' (within a virus strain or between viruses of different serotypes) with a higher fitness, which can therefore gain a potential selection advantage.

It would appear that the adaptive processes took several years following transmission of both avian H1N1 and human H3N2 viruses to European pigs. Following new introductions of influenza A virus to pigs, close monitoring of the epizootiology of SI in a population is essential to determine the rate of change, which, if elevated, may facilitate further transmissions across the species barrier with potential implications for disease control in a range of other species including humans. In future studies of the epidemiology of influenza viruses in pigs would be desirable to characterise all the gene segments of viruses isolated to detect changing genotypes with potential implications for pathogenicity to pigs and/or other species.

It is possible that following the transmission of an avian H1N1 virus to pigs in continental Europe in 1979 (Pensaert et al. 1981), subsequent infection of pigs was usually subclinical since the virus was not well adapted to its new host. It would appear that the introduction from continental Europe of an 'avian-like' swine H1N1 virus well adapted to its new host (Brown et al. 1997), into an immunologically naive pig population, such as found in GB in 1992, may partly explain the rapid spread of the virus and its widespread association with disease outbreaks (Brown et al. 1993), which was consistent with the epidemiology of the virus in

pigs in Europe as a whole. Analysis of viruses in Europe have revealed that is it possible for H1 viruses of different genetic characteristics to coexist within swine populations but equally there is also evidence that some strains lose fitness and become noncompetitive in such an environment and this can result in their ultimate extinction in pig populations (Brown 2008). Interestingly, immunity to the antigenically related classical swine H1N1 viruses of widespread prevalence in pigs in GB (Brown et al. 1995b) and continental Europe (Brown 2000) at the time, apparently failed to prevent infection with the newly emerged 'avian-like' swine H1N1 viruses.

The evolution and adaptation of human H3N2 viruses in pigs following transmission in the early 1970s appeared similar to that of avian H1N1 viruses. In Europe, the presence of these human H3N2 viruses in pigs was for at least 10 years based on antibody detection and it was not until 1984 that the virus was first associated directly with outbreaks of respiratory disease in pigs (Haesebrouck et al. 1985) and such occurrences became increasingly more frequent thereafter (Wibberley et al. 1988; Castrucci et al. 1994). Locally in many parts of Europe 'swine adapted' human H3N2 viruses became the predominant epidemic strain and still remain so for example in the 'Low countries' (De Jong et al. 1999; Van Reeth K, personal communication). Interestingly, H3N2 viruses circulating in pigs in Italy since 1983 all contain internal protein genes of avian origin, having replaced H3N2 viruses whose genes were derived entirely from human virus (Campitelli et al. 1997), suggesting that the acquisition of internal protein genes from an avian virus adapted to pigs afforded a selection advantage to these reassorted viruses.

Multiple exposures to different virus subtypes can lead to differential protective profiles and will depend on the sequence and combination of strains contained. It has been observed that pigs previously infected with H1N1 but not with H1N2 can develop cross reactive antibodies to H1N2 after vaccination (Van Reeth et al. 2006).

Antigenic drift occurs principally in the external glycoproteins of the virus, namely HA and NA. Analysis of H3N2 viruses isolated from pigs in the Netherlands and Belgium revealed in the early 1990s significant antigenic drift had occurred away from the progenitor strain A/Port Chalmers/1/73. In addition, significant drift antigenetically occurred in Italian viruses detected in the same period. Furthermore, these changes showed a cluster effect consistent with that reported for human viruses. Importantly, however, the evolutionary pathways between swine and human H1N2 viruses are clearly distinct, whereby the swine viruses in European pigs have progressively become more divergent from the contemporary human strains. It has been reported that this rate of antigenic evolution of swine viruses compared to human viruses in European pigs has occurred at a rate much slower than in humans (De Jong et al. 2007). The rationale for the lower rate of antigenic evolution in pigs is almost certainly linked to the fact that there is a continual supply of non-immune animals within a population and although long-lived pigs will acquire an immunity to infection which will influence the direction of the evolutionary selection process, this is slower than that which occurs in the human population. This in turn has a significant impact on the

changing epidemiology of influenza viruses in pigs. Following the initial emergence of H1N2 viruses in the UK in 1994, detailed genetic analysis of subsequent viruses have confirmed that viral genes of various origins have been stably maintained in pigs for many years before the multiple genetic reassortment was detected. Furthermore, this evolutionary analysis suggests that the HA and NA genes evolved with a significantly higher rate of non-synonymous substitutions after they were introduced from humans to pigs with subsequent establishment of a European H1N2 swine lineage (Tsan-Yuk et al. 2008).

The long term dynamic following the emergence of A (H1N1) pdm09 virus in European pigs will present some interesting observations on the complex interaction and fitness of endemic SI viruses despite distinct genetic differences, exposure to endemic swine H1 viruses in European pigs does provide a level of antigenic cross reactivity (Kyriakis et al. 2010) indicating that there will be some levels of prior immunity in pigs naturally exposed to endemic strains in the absence of pandemic virus. Furthermore, the impact upon transmission and shedding profiles in infected pigs is marked, with transmission being abrogated in animals that have prior immunity to avian-like swine viruses upon subsequent challenge with A(H1N1) pdm09 (Brookes et al. 2010). Evolutionary analysis of classical swine H1N1 and avian-like swine H1N1 viruses in European pigs has revealed that although there was clear lineage-specific bias there were also a number of certain functional and structural constraints on all of the genes segments that influence the long term evolutionary trajectory of viruses in pigs (Dunham et al. 2009).

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Swine Influenza Viruses: An Asian Perspective

Young-Ki Choi, Philippe Noriel Q. Pascua and Min-Suk Song

Abstract Swine influenza viruses (SIVs) are respiratory viral pathogens of pigs that are capable of causing serious global public health concerns in humans. Because of their dual susceptibility to mammalian and avian influenza A viruses, pigs are the leading intermediate hosts for genetic reassortment and interspecies transmission and serve as reservoirs of antigenically divergent human viruses from which zoonotic strains with pandemic potential may arise. Pandemic influenza viruses emerging after the 1918 Spanish flu have originated in Asia. Although distinct lineages of North American and European SIVs of the H1N1, H3N2, and H1N2 subtypes have been widely studied, less is known about the porcine viruses that are circulating among pig populations throughout Asia. The current review presents a historic account of the epidemiology and genetic evolution of SIVs in this region using data from the PubMed and Influenza Virus Resource databases. The current understanding of contemporary viruses, human infection with SIVs, and the potential threat of novel pandemic strains are described. Furthermore, to best use the limited resources that are available for comprehensive genetic assessment of influenza, consensus efforts among Asian nations to increase epidemiosurveillance of swine herds is also strongly promoted.

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Current Topics in Microbiology and Immunology (2013) 370: 147–172
DOI: 10.1007/82_2011_195

Published Online: 21 January 2011

1 Introduction

Influenza A virus is the culprit in recurrent annual influenza epidemics worldwide and has already caused catastrophic pandemics in humans. The segmented viral genome of this orthomyxovirus occurs as eight negative-sense, single-stranded RNAs that encode 11 functional proteins: the surface glycoproteins, Hemagglutinin (HA), and Neuraminidase (NA); the matrix proteins M1 and M2; the non-structural proteins NS1 and NS2; and the polymerase complex proteins PB2, PB1, PA, and nucleoprotein (NP). By virtue of antigenic variations of the surface glycoproteins, influenza A viruses can be subdivided into subtypes: 16 HA (i.e., H1–H16) and 9 NA (i.e., N1–N9) (Fouchier et al. 2005; Webster et al. 1992; Cheung and Poon 2007).

Although wild aquatic birds are the principal natural reservoirs of all HA and NA subtypes, influenza A viruses have also been isolated from a variety of other host species, including swine and humans (Alexander 1982; Webster et al. 1992). However broad their host range appears to be, these viruses are generally host specific and are not readily transmissible between species. But such restriction is not absolute because of error-prone replication, the segmented nature of the influenza A virus genome, and the probable involvement of intermediate hosts.

Apart from being one of the most common respiratory pathogens in swine and often causing economic burden in livestock-dependent industries, influenza infection in pigs is regarded as important in the evolution and ecology of influenza A viruses. Pigs are notoriously important intermediate hosts for interspecies transmission because of their dual susceptibility to human and animal influenza viruses. This susceptibility is due to sialyl oligosaccharide receptors lining their respiratory tract, which possess both *N*-acetylneuraminic acid-α2,3-galactose (preferred by avian influenza viruses) and N-acetylneuraminic acid-α2,6-galactose (preferred by mammalian influenza viruses) (Rogers and Paulson 1983; Ito et al. 1998). Apart from serving as sources for direct interspecies transmission of virus to people, swine are also known as "genetic mixing vessels" because of the reassortment of influenza viruses from various sources and lineages that takes place in infected pigs (Castrucci et al. 1993; Ma et al. 2008; Scholtissek 1990). Simultaneous infection of the same pig with different viruses could promote genetic reassortment that may significantly

alter viral evolution. Thus, pigs could be convenient hosts for the production of reassortant viruses with pandemic potential. Of the 3 influenza pandemics in the twentieth century, the 1957 (Asian flu) and 1968 (Hong Kong flu) pandemics were both caused by reassortant strains (i.e., H2N2 and H3N2, respectively) from avian and human viruses (Lindstrom et al. 2004; Scholtissek et al. 1978).

Although influenza is a winter disease in temperate and subarctic regions of the world, it is observed year round in tropical and subtropical parts of Asia, particularly China (Reichelderfer et al. 1989). Influenza viruses of the H1N1 and H3N2 subtypes are also prevalent in pigs year round in China and in some East and Southeast Asian nations (Guo et al. 1983; Shortridge and Webster 1979). Accordingly, southern China has long been considered to be an epicenter of pandemic influenza viruses (Shortridge and Stuart-Harris 1982). Due to the recent emergence of a swine-origin pandemic (H1N1) 2009 virus in North America, which rapidly spread globally, the significant role of pigs as focal points in the generation of pandemic viruses has once again been highlighted. In this report, we aim to review the genetic evolution and distribution of swine influenza viruses (SIVs) in Asia and their relation to their North American and European virus counterparts. Considering the possibility that pigs in Asia might usher future outbreaks and pandemics, we strongly recommend increasing the surveillance of influenza viruses in pig populations in this region.

2 History, Evolution, and Establishment of Swine Influenza Virus Lineages Among Pig Populations

Influenza A viruses of the subtypes H1N1, H1N2, and H3N2 circulate in major swine populations throughout the world (Brown 2000; Webby et al. 2004). However, the origin and antigenic and genetic characteristics of these SIV subtypes vary among different countries and continents (Olsen et al. 2005). Influenza was already recognized as a pertinent disease of swine observed in the United States, Hungary, and China as early as 1918, coinciding with the pandemic influenza virus of the H1N1 subtype in humans (Fig. 1; Chun 1919; Koen 1919; Beveridge 1977). However, it was not until the 1930s that the porcine virus was first isolated and identified in culture (Shope 1931).

It is thought that the pandemic virus of humans was also the causative strain that infected swine populations around the same time, as indicated by the close relatedness of the isolated strains, but these viruses had evolved separately in each host after the pandemic (Shope 1931). This swine virus, known as the "classical" H1N1 swine virus, continued to be found in pigs in the United States and Asia, but it was not found in Europe until 1980 (Pensaert et al. 1981; Peiris et al. 2009a). After the Hong Kong influenza pandemic in 1968, the wholly human-like H3N2–subtype virus (a non-reassorted H3N2 strain bearing segments identical to the virus circulating among humans) was transmitted to pigs and was initially detected in a Taiwanese slaughterhouse in 1969 (Kundin 1970; Shortridge et al. 1977). This originally avian–human reassortant virus continued to circulate in Europe and

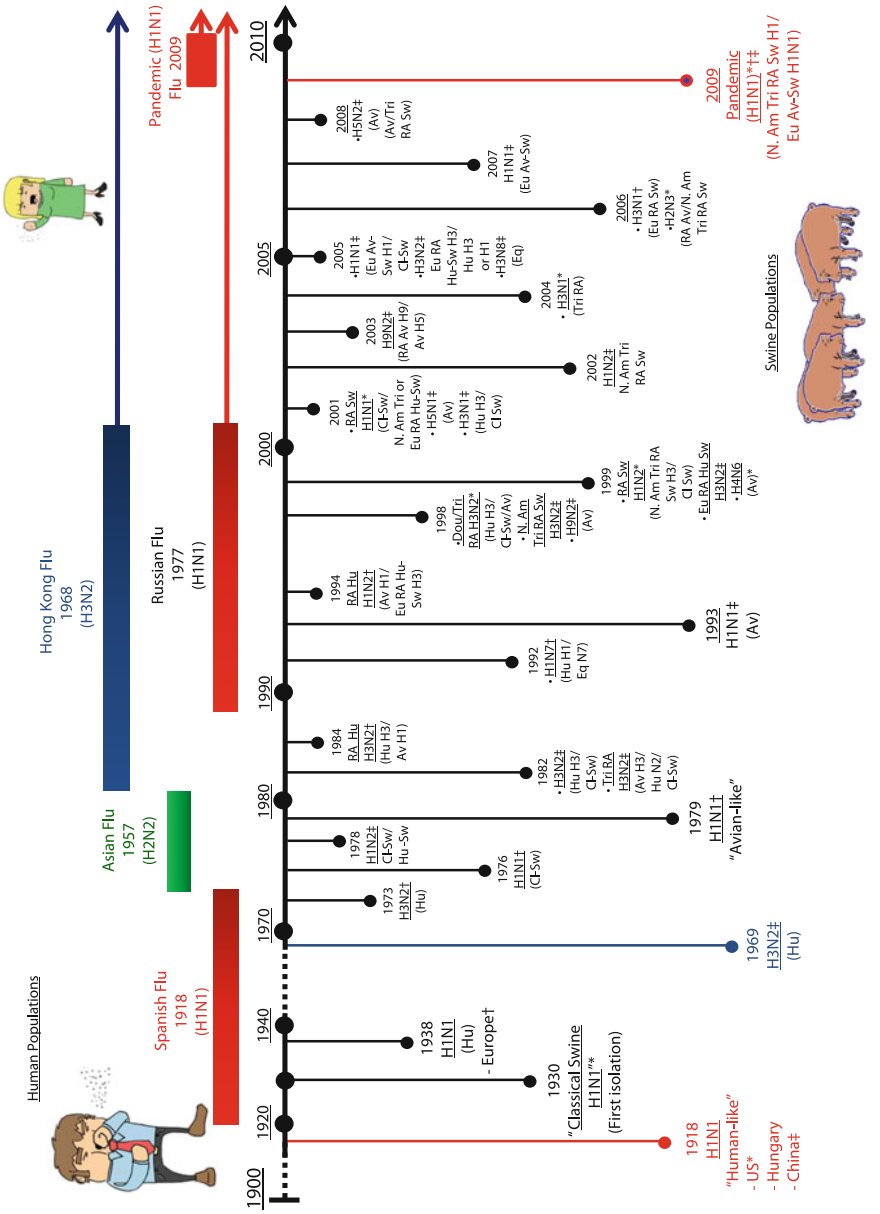

Fig. 1 Significant points in the history of swine influenza viruses from 1918 to 2010. The 1918 H1N1 Spanish Flu (*red bar*) and 1968 H3N2 (*blue bar*) Hong Kong Flu established lineages in pig herds. Interspecies transmission of influenza A viruses from avian and horse lineages, including novel reassortant viruses, were also isolated from pigs in North America (*asterisks*), Europe (*dagger*), and Asia (*double dagger*). 2009 marks the emergence and global spread of the pandemic (H1N1) 2009 virus. The italized entries denote appearance of atypical influenza virus strains in the different lineages. Virus origins (intact or recombinant) are indicated as: Av, avian-like; Av-Sw, Avian-like swine; Cl-Sw, classical swine-like; Eq, equine-like; Hu, human-like; Hu-Sw, human-like swine. Dou, double; Tri, triple; RA, reassortant

Asia; it was only infrequently found in North America before 1998, causing sporadic clinical signs (Chambers et al. 1991; Webby et al. 2000; Yu et al. 2008). The reemergent H1N1 virus in 1977 (Russian flu) that affected young adults and genetically closely resembled viruses of the early 1950s (Nakajima et al. 1978) was also isolated from pigs in Europe and Asia (Alexander 1982).

In North America, the "classical" swine H1N1 virus continued to be predominant over the sporadically detected Hong Kong H3N2 human virus in circulation among swine populations until 1998 (Hinshaw et al. 1978; Chambers et al. 1991; Karasin et al. 2000b). However, by late August 1998, two distinct genotypes of H3N2 influenza virus had emerged, caused outbreaks, and spread rapidly in North American swine populations (Zhou et al. 1999; Karasin et al. 2000c; Webby et al. 2000). These viruses were either double reassortant virus (i.e., HA, NA, and PB1 genes of human H3N2 and PB2, PA, NP, M, and NS genes of classical swine H1N1) or triple reassortant virus (i.e., human H3N2-classical swine H1N1 virus with PB2 and PA genes of a North American avian virus) (Zhou et al. 1999). Eventually, only the triple reassortant internal gene (TRIG) cassette virus continued to circulate and became established. Shortly after its emergence, cocirculation of this H3N2-reassortant virus with the previously predominant classical swine H1N1 virus resulted in the production of a reassortant H1N2 virus subtype that retained the entire TRIG backbone and acquired the HA gene of classical swine H1 (Karasin et al. 2000b). This lineage of reassortant H1N2 viruses has subsequently spread widely among North American pigs and is often associated with respiratory disease, although some strains have caused spontaneous abortion in sows (Choi et al. 2002a; Karasin et al. 2002). Reassortant H1N2 and H3N2 SIVs carrying the TRIG cascade have also been found in domestic turkey populations (Suarez et al. 2002; Choi et al. 2004a), and the H1N2 virus alone has been detected in wild waterfowl (Olsen et al. 2003; Ramakrishnan et al. 2010). In humans, 11 patients in the United States have been confirmed to be infected with triple-reassortant H1 SIVs since 2005 (Shinde et al. 2009). The following swine viruses containing the original TRIG genetic constellation and differing only in surface glycoproteins continue to circulate in North American swine populations: three distinct H3N2 viruses (each with a human-like HA from a different year), one H1N2 virus with a human-like HA, one H1N2 virus with a classical swine-like HA, one H1N1 virus with a human-like HA and NA, and one H1N1 virus with a classical swine-like HA and NA (Fig. 2a; reviewed by Brockwell-Staats et al. 2009). Some of these viruses have also been found in Asian pigs.

SIVs in European pigs have undergone a more divergent evolutionary pattern than their North American lineage counterparts. Viruses in Europe differ in their origin (i.e., transmission from birds or from humans, either wholly or after single or multiple reassortment events) from those found on other continents, and various lineages can be distinguished within each subtype (Kuntz-Simon and Madec 2009). Although H1N1 influenza viruses were isolated or detected earlier in a few European nations, it was not until 1976 that the classical swine H1N1 virus became clearly established and endemic in pigs throughout Europe (Brown 2000). Approximately a year later, human-like H1N1 viruses reemerged among European

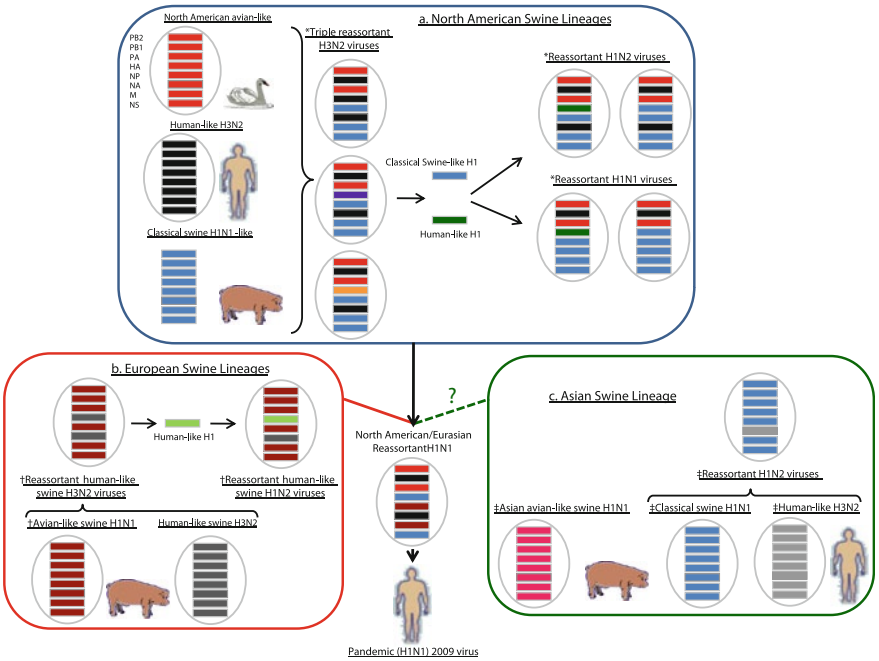

Fig. 2 Genotypes of H1N1, H3N2, and H1N2 swine influenza viruses circulating worldwide. Distinct lineages of swine influenza viruses continue to be endemic in pig herds worldwide. North American swine viruses (*blue box*, *asterisks*) are composed of three distinct H3N2 viruses (each with a human-like HA from a different year), an H1N2 virus with a human-like HA, an H1N2 virus with a classical swine-like HA, an H1N1 virus with a human-like HA and NA, and an H1N1 virus with a classical swine-like HA and NA (Brockwell-Staats et al. 2009). Circulating European lineage swine viruses (*red box*, *dagger*) are composed of avian-like swine H1N1, reassortant human-like swine H3N2, and various genotypes of reassortant human-like swine H1N2 viruses (Kuntz-Simon and Madec 2009). Although avian-like H1N1, classical swine H1N1, human-like H3N2, and unique reassortant H1N2 viruses have been found among Asian pig herds (*green box*, *double dagger*), the prevalence of swine viruses in major parts of Asia is unknown. A triple-reassortant swine virus recently reassorted with a Eurasian avian-like swine virus, producing a novel pandemic (H1N1) 2009 virus that has circulated in humans since April 2009. However, the precise viral precursor or when and where the causative pandemic virus arose still remains unknown

pigs subsequent to the so-called Russian flu that began among humans in China during the winter of 1977–1978 (Nakajima et al. 1978; Alexander 1982; Kuntz-Simon and Madec 2009). Wholly human-like H3N2 viruses, even long after their disappearance from the human population, also emerged in European swine since the 1970s and continued to circulate for about 10 years causing sporadic clinical outbreaks (as reviewed by Kuntz-Simon and Madec 2009). In 1979, the emergence of the wholly avian-like H1N1 virus in Italy marked the displacement of the classical swine as the dominant stable H1N1virus lineage among swine populations in Europe (Pensaert et al. 1981; Campitelli et al. 1997; Scholtissek et al. 1983). Reassortment between these avian-like H1N1 SIVs and cocirculating

human-like H3N2 viruses generated reassortant human-like swine H3N2 virus (i.e., human-like surface genes and internal genes of avian origin) in 1984 (Campitelli et al. 1997), which replaced the original human-like swine H3N2 strain in circulation among pig populations in many European countries in the 1990s (Kuntz-Simon and Madec 2009). Likewise, further reassortment between the avian-like H1N1 virus and the reassortant human-like H3N2 in 1987 produced the first reported European SIV isolate of the H1N2 subtype; this isolate featured HA and NA genes of avian and human origins, respectively (Gourreau et al. 1994). The virus, which did not establish a stable lineage, was succeeded by another novel H1N2 virus that quickly spreads to Western Europe (i.e., UK, Belgium, France, Italy, Germany, and Spain) (Kuntz-Simon and Madec 2009). This reassortant human-like H1N2 virus is quite similar in gene component to the European reassortant human-like H3N2 virus except for the HA surface glycoprotein (human-like H1 and N2 with avian-like internal genes) (Fig. 2b). From then on, variants of H1N2 strains had been reported in several parts of Europe, most of which contained human-like HA and NA genes with the internal protein genes derived from European avian-like swine H1N1 viruses (Marozin et al. 2002; Hjulsager et al. 2006; Chiapponi et al. 2007; Franck et al. 2007; Zell et al. 2008; Bálint et al. 2009). Few of these European swine viruses have also been found circulating in Asian pig herds.

3 Swine Influenza in Asia

The circulation of SIVs in Asia is more complex than it is elsewhere. Some SIVs of the North American and European lineages described above have been frequently detected in Asia, likely because of the intercontinental importation of live pigs from these two regions. Apart from these viruses, there are also several lineages that are found only in Asia. Table 1 provides an overview of the occurrence of diverse SIVs in Asia.

3.1 H1N1 Virus Strains

The classical swine H1N1 virus, which evolved during the "Spanish influenza" pandemic was first observed in Chinese pigs in 1918 (Fig. 1; Chun 1919). After the initial occurrence, the virus, or at least its antibodies, was also detected in Hong Kong, Japan, India, Taiwan, Singapore, Iran, Thailand, Korea, and Malaysia (Yip 1976; Yamane et al. 1978; Das et al. 1981; Shortridge and Webster 1979; Samadieh and Shakeri 1976; Kupradinun et al. 1991; Lyoo and Kim 1998; Mohamed et al. 2010). As the spread of Russian (H1N1) influenza viruses from humans to pigs was documented in the late 1970s in some European countries, a case was also observed from a diseased pig in Thailand (Nerome et al. 1982b).

Table 1 Records of swine influenza virus detection and isolation in Asia

Country	Subtype	Year initially isolated or serologically detected	Origin	Swine genetic lineage	Reference
China	H1N1	1918 (Serology)	Hu		Chun (1919)
		1979 (Serology)	Classical Sw		Shortridge and Webster (1979)
		1993	Av		Guan et al. (1996)
		2006	Hu		Yu et al. (2007)
		2007	Av-like Sw	European	Liu et al. (2009a)
	H1N2	2001	Classical Sw/ reassortant Sw	European/Asian	Smith et al. (2009)
		2004	Classical Sw/Hu		Qi and Lu (2006)
		2004	Reassortant Sw/ Sw	North American/ Eurasian	Smith et al. (2009)
		2006	Reassortant Sw/Sw	North American/Asian	Yu et al. (2009b)
	H3N2	1976 (Serology)	Hu		Shortridge et al. (1977), Shortridge and Webster (1979)
		1982	Hu/Classical Sw		Nerome et al. (1995)
		1982	Hu/Classcical Sw/ Av	Asian	Yu et al. (2008)
		1999	Reassortant Sw	European	Yu et al. (2008)
		2001	Reassortant Sw/Av	European/Asian	Yu et al. (2008)
	H3N8	2005	Equine		Tu et al. (2009)
	H5N1	2001	Av		Li et al. (2004); Zhu et al. (2008)
	H9N2	1998	Av		Peiris et al. (2001), Cong et al. (2007)
		2003	Av/Av		Xu et al. (2004), Shi et al. (2008)

(continued)

Table 1 (continued)

Country	Subtype	Year initially isolated or serologically detected	Origin	Swine genetic lineage	Reference
Japan	H1N1	1977 (Serology)	Classical Sw		Yamane et al. (1978), Nerome et al. (1982a)
		1992	Hu		Katsuda et al. (1995)
	H1N2	1978	Classical Sw/Hu		Sugimura et al. (1980)
	H3N2	1969 (Serology)	Hu		Nerome et al. (1981)
Korea	H1N1	1996	Classical Sw		Lyoo and Kim (1998)
		2003	Reassortant Sw	North American	Choi et al. (2004a)
	H1N2	2002	Reassortant Sw	North American	Choi et al. (2002b)
	H3N1	2006	Reassortant Sw/ Classical Sw	North American	Shin et al. (2006)
	H3N2	1995	Hu		Lyoo and Kim (1998)
		1998	Reassortant Sw	North American	Song et al. (2003), Pascua et al. (2008)
	H5N2	2008	Av		Lee et al. (2009)
		2008	Av/Sw		Lee et al. (2009)
India	H1N1	(Serology)	Hu		Das et al. (1981)
	H3N2	1970s (Serology)	Hu		Singh et al. (1979)
Indonesia	H3N1	2004	Hu/Classical Sw		Chan et al. (2010)
	H5N1	2006	Av		Mahardika (2008), Takano et al. (2009)
Malaysia	H1N1	1984	Classical Sw		Mohamed et al. (2010)
		2005 (Serology)	Sw	European	Suriya et al. (2008)
	H3N2	2005 (Serology)	Sw	European	Suriya et al. (2008)

(continued)

Table 1 (continued)

Country	Subtype	Year initially isolated or serologically detected	Origin	Swine genetic lineage	Reference
Singapore	H1N1	1979 (Serology)	Classical Sw		Shortridge and Webster (1979)
Taiwan	H1N1	1977 (Serology)	Classical Sw		Shortridge and Webster (1979)
	H1N2	2004	Hu/Sw		Shieh et al. (2008)
	H3N1	2001	Hu/Classical Sw		Tsai and Pan (2003)
	H3N2	1969	Hu		Kundin, (1970)
Thailand	H1N1	1979	Hu		Nerome et al. (1982b)
		1988	Classical Sw		Kupradinun et al. (1991)
		2000	Reassortant Sw/Av	European/Asian	Takemae et al. (2008)
	H1N2	2005	Reassortant Sw/Sw	European/Asian	Chutinimitkul et al. (2008)
	H3N2	1978	Hu		Nerome et al. (1981)
		2003	Reassortant Sw	European	Takemae et al. (2008)
Vietnam	H5N1	2004 (serology)	Av		Choi et al. (2004a)

Epidemiology of SIVs in Asia was obtained using data from the PubMed and Influenza Virus Resource databases (Bao et al. 2008). *Av* avian, *Hu* human, *Sw* swine

By 1993, avian-like H1N1 influenza viruses had been isolated from clinically healthy pigs in southern China and were circulating with classical H1N1 viruses (Guan et al. 1996; Yu et al. 2009b). Emergence of these viruses was independent from the introduction and establishment of the avian-like H1N1 strain in European pigs but phylogenetically formed an Asian sublineage of the Eurasian avian virus lineage (Brown 2000). Intact European avian-like H1N1 SIVs infiltrated swine populations in Fujian, Shandong, and Beijing provinces of China in 2007 (Liu et al. 2009a) without causing any signs of disease. Non-pandemic human-like H1N1 viruses have also been sporadically isolated from pigs in Japan (Katsuda et al. 1995) and, more recently, in China (Yu et al. 2007, 2009a).

Several reassortant swine H1N1 viruses of either the North American or European lineage could also be observed circulating in Asian pigs. Porcine isolates from Korea, which are genetically identical to North American reassortant H1N1 viruses bearing classical swine-like HA and NA, have been continually isolated since 2003 (Choi et al. 2004a; Song et al. 2003; Pascua et al. 2008). In contrast, circulating H1N1 viruses from pigs in Thailand contained a mixture of genes from classical swine and Eurasian avian-like swine H1N1 lineages (Chutinimitkul et al. 2008; Takemae et al. 2008). Serosurveillance among pig farms of Peninsular Malaysia from 2005 to 2007 indicated the presence of H1N1 strains that are probably European-like viruses, although the complete genetic description was not reported (Suriya et al. 2008). Classical swine H1N1 viruses continue to be endemic in southern China and Southeast Asia (Guan et al. 1996; Peiris et al. 2009a; Qi et al. 2009).

3.2 H3N2 Virus Strains

The wholly human-like H3N2 virus was first isolated from slaughterhouse pigs of Taiwan in 1969 after the Hong Kong human pandemic a year earlier (Kundin 1970). The existence of this new virus provided evidence that prevailing human subtypes could infect pigs under natural conditions (Brown 2000). Studies to examine the potential transmission of human strains to pigs showed that antibodies to the human-like H3N2 viruses could be detected in pigs from Taiwan (Kundin 1970), China (Shortridge and Webster 1979), India (Singh et al. 1979), and Korea (Lyoo and Kim 1998) and had reached widely separate regions in Europe and the United States (Sect. 2). Virus isolations were also reported in Hong Kong, Japan, and Thailand (Shortridge et al. 1977; Nerome et al. 1981). From then on, evolution of H3N2 SIVs in Asia has been quite complex, most notably in Southeast Asia. H3N2 swine viruses that contained human-like HA and NA and classical swine-like internal gene segments were isolated in China in the early 1980s (Shu et al. 1994; Nerome et al. 1995). Similarly, around the same time, two triple-reassortant H3N2 viruses, genetically unrelated to those of the North American or European reassortant viruses, were also isolated from Chinese pigs. One of the isolates (i.e., A/Sw/Hong Kong/126/82) contained gene segments similar to those of the avian-like (HA, PB1, PA, and NP), the human-like (NA), and the classical

swine-like (PB2, M, and NS) lineages, whereas the other isolate (A/swine/Hong Kong/127/82) contained genes from the human-like (HA and NA), classical swine-like (PB2, PB1, PA, NP, and NS), and avian-like (M) lineages (Yu et al. 2008). After more than a decade, European reassortant human-like swine H3N2 viruses emerged among pigs in China in 1999. Approximately 2 years later, further reassortment of these viruses with circulating classical swine H1N1 viruses that donated the NP gene produced triple-reassortant strains (Yu et al. 2008). In Thailand, these European reassortant human-like swine H3N2 viruses also genetically recombined with circulating domestic human-like H1 or H3 strains (Takemae et al. 2008; Chutinimitkul et al. 2008). Antibodies against these European viruses were detected in Malaysian pigs (Suriya et al. 2008). SIV strains isolated from Korea since 1998 represent all the triple-reassortant H3N2 viruses of the North American swine lineage, which have three distinct human-like HAs (Song et al. 2003; Pascua et al. 2008). Pigs from which these H3N2 virus strains were isolated had respiratory disease signs typical of influenza in pigs, but none of the illnesses resulted in spontaneous abortion or death.

3.3 Reassortant H1N2 Virus Strains

Reassortant influenza A H1N2 viruses, derived from human-like swine H3N2 and classical swine H1N1 viruses, were first isolated in Japan in 1978 (Sugimura et al. 1980) and became endemic in Japanese swine populations, establishing a genetically stable lineage (Yoneyama et al. 2009). A similar gene cassette of Asian reassortant H1N2 viruses (Fig. 2c) has been found circulating in Taiwanese pig herds (Tsai and Pan 2003) and in a 2004 isolate in Zhejiang province, China (Qi and Lu 2006). In Korea, North American reassortant H1N2 viruses containing classical swine-like HAs have been isolated since 2002 (Choi et al. 2002b). From then on, successive H1N2 SIVs formed a genetically stable lineage that spread widely within Korean swine populations (Pascua et al. 2008), a feat similar to that of the Japanese H1N2 SIVs.

H1N2 reassortant viruses with classical swine H1 and Eurasian avian-like swine lineage for the remaining genes were isolated from pigs in Thailand (Chutinimitkul et al. 2008; Takemae et al. 2008). A genetically similar SIV was also isolated in 2004 from a 25-year-old man in the Philippines who had mild influenza (Komadina et al. 2007). However, it is not certain whether or not this isolate represents strains that are currently circulating in domestic pigs in the Philippines because very little is known about the local epidemiology of SIVs there. More diverse genotypes of reassortant H1N2 viruses have also been observed in China, particularly in the southeast regions, since 2001. The majority of these reassortant H1N2 SIVs were the product of genetic recombination between contemporary circulating classical swine H1N1 viruses and European reassortant or North American triple reassortant viruses (Smith et al. 2009; Yu et al. 2009b).

3.4 Interspecies Transmission and Novel Influenza Virus Strains

Most pandemic strains (of the H1N1 and H3N2 subtypes) since the 1918 Spanish flu have been detected in major pig populations in North America, Europe, and Asia shortly after their appearance and circulation in humans. It appears that only the Asian pandemic virus (H2N2) in 1957 was unable to be transmitted to swine. Interspecies transmission of avian viruses to pigs has also been continually observed on these continents. In fact, reassortant swine viruses that are currently circulating have retained at least one avian-like viral gene component. Atypical and novel viruses of the H1N7 and H3N1 subtypes in Europe (Brown et al. 1997; Moreno et al. 2009) and the H4N6, H3N3, H3N1, and H2N3 subtypes in North America (Karasin et al. 2000a, 2004; Ma et al. 2007; Lekcharoensuk et al. 2006) have been sporadically isolated from pigs (Fig. 1). However, none of these has successfully established a stable lineage in swine populations.

Since 1998, several avian Dk/Hong Kong/Y280/97-like H9N2 viruses have been isolated from pigs in Hong Kong and the provinces of Shandong and Henan in China (Peiris et al. 2001; Cong et al. 2007, 2009). Phylogenetically, these viruses do not form a single lineage, implying that multiple interspecies transmission events of H9N2 viruses from avian hosts to pigs have naturally occurred in China (Cong et al. 2009). Although most of the earlier porcine H9N2 isolates were wholly avian-like (i.e., unreassorted), some viruses since 2003 have been reassortant avian-like strains with some genes closely related to highly pathogenic avian influenza (HPAI) H5N1-like viruses (Xu et al. 2004; Shi et al. 2008).

During routine surveillance of influenza virus activity, HPAI H5N1 viruses were isolated from pigs in southern (i.e., Fujian province) and eastern (i.e., Shandong province) China in 2001 and 2003, respectively (Zhu et al. 2008; Li et al. 2004; Shi et al. 2008). One of the porcine H5N1 isolates from Fujian was relatively attenuated in mice; this was attributed to a 15-nucleotide deletion in the NS gene that impaired the ability of the virus to antagonize interferon (IFN-α and IFN-β) responses, affected the stability of the NS1 protein, and impaired interaction with the cleavage and polyadenylation specificity factor protein (Zhu et al. 2008). Remarkably, another Fujian H5N1 isolate, which does not bear the characteristic base deletions, was lethal in mice at high doses despite the absence of molecular markers in its PB2 protein that are associated with enhanced replication and virulence in mice models (Zhu et al. 2008). Infection with avian-like H5N1 viruses was also found in asymptomatic pigs from Indonesia (Mahardika 2008; Takano et al. 2009) and Vietnam (Choi et al. 2004c).

H3N1 SIVs have been isolated from pigs in Taiwan, Indonesia, and Korea (Tsai and Pan 2003; Chan et al. 2010; Shin et al. 2006). The Taiwanese strains appear to be reassortant virus with genes of the human-like H3N2 isolate and

Table 2 Worldwide animal infections with the pandemic (H1N1) 2009 virus

Location	Host	Date reported	Reference
North America			
[a]Canada (Alberta)	Swine	May 2009	Howden et al. (2009)
Canada (Ontario)	Turkey	October 2009	OIE (2010 Vol. 22—No. 44)
Canada (Manitoba)	Swine	June 2009	Pasma and Joseph (2010)
US (Oregon)	Ferret	October 2009	AVMA (2010)
US (Minnesota)	Swine	October 2009	AVMA (2010)
US (Alaska)	Ferret	October 2009	AVMA (2010)
US (Indiana)	Swine	November 2009	OIE (2010 Vol. 22—No. 45)
US (Iowa)	Cat	November 2009	AVMA (2010)
US (Utah)	Cat	November 2009	AVMA (2010)
US (Virginia)	Turkey	December 2009	OIE (2010 Vol. 22—No. 49)
US (California)	Cheetah	December 2009	AVMA (2010)
US (Colorado)	Cat	December 2009	AVMA (2010)
US (Pennsylvania)	Cat	December 2009	AVMA (2010)
US (New York)	Dog	December 2009	AVMA (2010)
US (North Carolina)	Swine	December 2009	AVMA (2010)
US (California)	Cat	December 2009	AVMA (2010)
US (California)	Turkey	January 2010	AVMA (2010)
South America			
Chile	Turkey	August 2009	OIE (2010 Vol. 22—No. 35)
Argentina	Swine	June 2009	Pereda et al. (2010)
Mexico	Swine	December 2009	OIE (2010 Vol. 22—No. 50)
Europe			
United Kingdom	Swine	September 2009	OIE (2010 Vol. 22—No. 39)
[a]Ireland	Swine	October 2009	OIE (2010 Vol. 22—No. 40)
[b]Norway	Swine	October 2009	Hofshagen et al. (2009)
Iceland	Swine	October 2009	OIE (2010 Vol. 22—No. 44)
Finland	Swine	December 2009	OIE (2010 Vol. 22—No. 49)
France	Cat	December 2009	AVMA (2010)
Italy	Swine	December 2009	OIE (2010 Vol. 22—No. 50)
Germany	Swine	December 2009	OIE (2010 Vol. 22—No. 50)
Russia	Swine	December 2009	OIE (2010 Vol. 22—No. 52)
Denmark	Swine	January 2010	OIE (2010 Vol. 23—No. 2)
France	Turkey	January 2010	OIE (2010 Vol. 23—No. 3)
Serbia	Swine	January 2010	OIE (2010 Vol. 23—No. 4)
Australia			
Canberra	Swine	August 2009	OIE (2010 Vol. 22—No. 32)
Asia			
Singapore	Swine	September 2009	Kyriakis et al. (2010)
Japan	Swine	October 2009	OIE (2010 Vol. 22—No. 43)
Taiwan	Swine	November 2009	OIE (2010 Vol. 22—No. 45)

(continued)

Table 2 (continued)

Location	Host	Date reported	Reference
Indonesia	Swine	November 2009	OIE (2010 Vol. 22—No. 48)
China	Dog	November 2009	AVMA (2010)
	Swine	December 2009	AVMA (2010)
Thailand	Swine	December 2009	OIE (2010 Vol. 22—No. 51)
Korea	Swine	December 2009	OIE (2010 Vol. 22—No. 52)

[a] Personnel involved in handling or sampling of pigs on the index outbreak developed influenza-like illnesses and were confirmed positive for pandemic influenza H1N1 2009
[b] Pig-to-pig transmission of A H1N1 within the same herd was highly suspected after a high proportion (18/20) of pigs sampled from the index farm tested positive
AVMA American Veterinary Medical Association, *OIE* Office International des Epizooties

circulating classical H1N1 SIVs (Tsai and Pan 2003). Although the Indonesian isolate appears to have a similar gene cassette, it is uncertain whether or not this virus typifies the circulating porcine viruses in domestic pigs because there are no previous records of H3 or H1 strains in Indonesia for comparison. Furthermore, low maximum sequence homologies in HA (85%) and NA (89%) genes suggest that this isolate may not be related to the Taiwanese isolate. In Korea, reassortment between co-circulating reassortant H1N1 and H3N2 viruses of the North American lineage was responsible for the genesis of H3N1 SIVs (Shin et al. 2006). In China, wholly equine-like H3N8 viruses were also obtained from pigs in central China that were showing signs of respiratory disease and depression (Tu et al. 2009).

Recently, a wholly avian-like H5N2 virus was directly transmitted from wild migratory birds into Korean pigs (Lee et al. 2009). It appears that, after coinfection with the avian virus and an H3N1-like SIV, the viruses reassorted in the pig to produce an avian-swine–like recombinant virus in which the PB2, PA, NP, and M genes were replaced by the corresponding segments from the swine strain. Possession of these segments allowed the transmission of the reassortant H5N2 virus in pig models but not in ferrets (Lee et al. 2009). Retrospective serosurveillance also indicated that avian-like H5 viruses have been infecting Korean swine herds since 2006 (Lee et al. 2009).

Most of the viruses reported here appear to have only a limited, sporadic existence in swine, as no subsequent isolations were reported after each index case (Table 1). However, a pooled data analysis of serologic surveillance data gathered from different provinces of China from 1999 to 2009 revealed that H5- and H9-like viruses are still circulating in pig populations, albeit with low prevalence (Liu et al. 2009b). Furthermore, although most of these viruses were isolated from mildly diseased pigs with typical influenza-like illness, some reassortant porcine H9N2 viruses possessing avian-like H5N1 genes resulted to swine deaths (Xu et al. 2004). In contrast, pig infections with wholly HPAI H5N1 virus were relatively asymptomatic (Takano et al. 2009; Li et al. 2004; Zhu et al. 2008).

3.5 Zoonotic Transmission of Swine Viruses in Asia

The isolation of a swine-origin influenza virus in 1974 from a diseased patient provided the first confirmation of the zoonotic nature of SIVs (Smith et al. 1976).There are only three reports of human infection with SIVs in Asia in the PubMed database. In September 1999, an influenza A H3N2 virus was isolated from a 10-month-old girl who had a fever and congested throat and was admitted to a hospital in Hong Kong (Gregory et al. 2001). The isolate was antigenically and genetically identical to H3N2 SIVs that were prevalent in European pigs during the 1990s, particularly to viruses that had infected two children in the Netherlands in 1993 (Claas et al. 1994). An H1N2 virus was isolated in a mildly diseased 25-year-old man in the Philippines in 2004, and an H1N1 isolate was obtained from a 4-year-old boy in Thailand who was suffering from a fever, rhinorrea, and myalgia (Komadina et al. 2007). These two viruses also appear to be closely related to reassortant Eurasian H1 SIVs. The Thailand isolate was similar to H1N1 SIVs circulating in domestic pigs (Chutinimitkul et al. 2008). None of the patients had any known exposure or direct contact with pigs, and all of them recovered upon treatment (Gregory et al. 2001; Komadina et al. 2007). No further reports of such infections were identified, including among the patients' family members, indicating a lack of horizontal transmission.

Diverse lineages of H1N1, H3N2, and to a lesser extent H1N2 SIVs have been zoonotically transmitted to humans around the world (Van Reeth 2007; Myers et al. 2007; Komadina et al. 2007). Considering the two human isolates found by Komadina et al. (2007) and the retrospective analysis by Myers et al. (2007), the majority of influenza-like illnesses that are due to apparent zoonotic infections with SIVs since 1958 have been mild in nature, but approximately 13.5% of the 52 cases worldwide were fatal. However, this figure may not necessarily reflect the true global incidence of natural infection with SIVs among human populations.

4 Emergence of Swine-Origin Pandemic H1N1 (2009) Virus

As previously discussed, circulating North American swine H1 viruses since 1998 have been triple-reassortant strains. The high capacity of these viruses to reassort with cocirculating viruses has generated various reassortants that are occasionally transmitted to humans but fail to extend infection beyond index cases (Brockwell-Staats et al. 2009). However, the seemingly restricted and benign nature of these viruses toward human populations changed when they acquired the NA and M gene segments of Eurasian-like H1N1 SIVs through reassortment (Trifonov et al. 2009; Garten et al. 2009). After the zoonotic transfer of this novel avian-swine-human reassortant virus in April 2009 (Dawood et al. 2009) and the consistent human-to-human transmission, a new influenza pandemic was declared nearly 2 months later by the World Health Organization (WHO) (Cohen and

Enserink 2009). Nonetheless, overall index morbidity and mortality has been considered lower than those seen during severe seasonal influenza outbreaks (Peiris et al. 2009b). As of 30 May 2010, more than 18,000 deaths had been reported globally (WHO 2010).

The emergence of the pandemic (H1N1) 2009 virus is also a classic example of the role of pigs as reservoirs for old virus strains for subsequent infection of human populations (Castrucci et al. 1994). Serologic investigations and virus challenge simulation studies showed that contemporary seasonal human influenza vaccines could provide little, if any, protection against infection with the pandemic (H1N1) 2009 virus (Centers for Disease Control and Prevention 2009; Kelly and Grant 2009; Pascua et al. 2009), making strain-specific vaccines necessary. Although contemporary seasonal influenza H1N1 virus and most currently circulating swine H1 viruses are descendants of the same 1918 H1N1 virus, separate evolution in human and swine hosts ultimately resulted in antigenic divergence.

As the new pandemic virus continued to spread among human populations worldwide, sporadic detection and isolation in various animals in different countries had also gradually increased, most importantly among swine herds. Table 2 provides a summary of reports on animal infections with the pandemic (H1N1) 2009 virus on a global scale, which includes breeding turkeys, cheetahs, and household pets such as dogs, cats, and ferrets. Because the virus hypothetically arose from pigs, it is natural that reverse zoonoses occurs, as already proved in experimental and natural conditions. Therefore, it is also likely that the virus will be established among swine herds. In Asia, the continued endemic potential of HPAI H5N1 viruses in poultry and swine poses a risk for reassortment. Turkeys readily infected with the pandemic (H1N1) 2009 virus also provide an alternative host to converge and recombine with other avian influenza viruses.

5 Discussion

SIV is a very important zoonotic disease that causes serious public health problems and economic burdens on a global scale. Influenza viruses of the subtypes H1N1, H3N2, and H1N2 have already been concurrently circulating among swine populations in most parts of Europe and North America. However, the antigenic and genetic makeup of SIVs in Europe differ significantly from those viruses currently found in North America (Kothalawala et al. 2006), establishing separate lineages of influenza viruses from swine. Furthermore, different epizootiological patterns have been seen in various swine populations worldwide. Except from the eastern and southeastern regions, which include China, Taiwan, Japan, Korea, and Thailand, extremely limited information is available about the epidemiology of SIVs from greater parts of Asia. Classical swine H1N1 viruses continue to circulate among pigs in southeast Asia and have cocirculated with avian-like swine H1N1 viruses in China since 1993 (Fig. 2c) (Guan et al. 1996). Human H3N2 viruses, first isolated from pigs in Taiwan in

1969 (Kundin 1970), are also found in swine herds along with several different reassortant H3N2 viruses (Table 1). Subsequent reassortment between cocirculating human-like H3N2 and classical swine H1N1 viruses led to the appearance of reassortant swine H1N2 viruses that are unique from the H1N2 viruses of other lineages. In addition to these Asian viruses, most of the European swine viruses (i.e., avian-like H1N1 and reassortant human-like H3N2 viruses) have also been found in Asia and often undergo reassortment with locally circulating swine viruses (e.g., in Thailand) or contemporary human-like H3N2 and H1N1 viruses (e.g., in China), generating several different reassortant strains (Chutinimitkul et al. 2008; Takemae et al. 2008; Yu et al. 2008). These Asian isolates, which bear segments akin to European viruses, are collectively termed "Eurasian-like." In addition, North American-like lineage swine viruses have reassorted with contemporary Eurasian SIVs in Chinese pigs (Smith et al. 2009), although they have been isolated exclusively from Korean swine populations (Pascua et al. 2008). Although isolation or seroprevalence reports of SIVs have been made in a few other Asian countries, the more comprehensive genetic data and analysis that are needed to better understand the complete genetic relation of the circulating viruses in Asia are lacking.

Although pigs are commonly domesticated animals found throughout the world, their incidence and consumption in Asia is greatly influenced by social customs and climate or bound by strict adherence to religious decrees (Webster et al. 1992). Therefore, the prevalence of porcine influenza viruses in this region may also be affected. Swine husbandry practices directly affect the evolution of influenza viruses in pigs (Brown 2000). Large-scale pig husbandry might be practiced by some Asian swine farms, but most independent pig raisers practice small-scale holdings and, often, animals are reared together or near poultry farms tended by similar workers. It is in these small-holding facilities, where close interaction between pigs, poultry, and humans continues, that the opportunity for interspecies transmission, including genetic recombination, of influenza viruses is greatest (Shortridge and Stuart-Harris 1982). Such transmission has been observed in southern China, and it is, therefore, regarded as an "influenza epicenter" (Shortridge and Stuart-Harris 1982), a potential source of the next human influenza pandemic. Isolation of atypical and novel subtypes has been considerably more frequent from Asian pigs than from their European or North American counterparts.

Prior to the pandemic (H1N1) 2009 virus in humans, SIVs had not caused any significant global outbreak of influenza in humans. Prospective studies on the real impact of zoonotic infection of SIVs in Asia are lacking. As mentioned earlier, there are only three reports of human infections with Asian SIVs in which porcine-like influenza viruses were characterized, likely by chance; this does not reflect accurate animal-to-human transmission rates. Such inadequate information indicates the under-appreciation of SIVs by authorities in some Asian countries. In a large continent where many of the nations are not affluent and such public health concerns are not of top priority, the lack of significant surveillance data might only be a matter of overlooked importance. The prevailing sociocultural practices and weak public health infrastructure in developing countries of Asia further enhances

the vulnerability of the region as the epicenter of outbreaks due to zoonotic infections (Bhatia and Narain 2010). The role of people working with and constantly being exposed to swine in the epidemiology of SIVs has been recently acknowledged (Gray et al. 2007). Aside from having a high risk of being the first to be infected in the event that a novel virus becomes epizootic in swine herds, these workers may serve as a bridge for transmission of the virus to their communities, consequently accelerating the occurrence of pandemics (Gray et al. 2007; Myers et al. 2007).

Of the numerous genotypes of H1N2 viruses in Asia (derived from multiple reassortment events) that have been identified, a reassortant virus from Hong Kong in 2004 contained five internal genes from the North American triple reassortants and had Eurasian-like lineage H1 and M segments (Smith et al. 2009). Another H1N2 reassortant virus isolated in Thailand also contained a classical swine H1 gene, and its remaining genes were from Eurasian avian-like swine (Chutinimitkul et al. 2008; Takemae et al. 2008). Epidemiologically speaking, it is very tempting to speculate that these two viruses might represent possible intermediary viruses in a sequential process of reassortment events that led to the genesis of the swine-origin pandemic (H1N1) 2009 strain (Fig. 2). However, surveillance data of SIVs in Central and South America, where the pandemic virus emerged, are still lacking (Brockwell-Staats et al. 2009). Therefore, any interpretation is still merely speculative. Establishing where the reassortment event took place may remain a dilemma for experts, and the hypothetical precursors may never be resolved, similar to what happened with the H2N2 and H3N2 pandemics. The most pressing concern is whether the pandemic virus will be established in pig populations worldwide and finds ways to reassort with avian viruses or drug-resistant human strains. Establishment in pig herds is apparently very likely because the pandemic (H1N1) 2009 virus technically arose from pigs, and there are reported cases of reverse zoonoses from various countries (Table 2). In experimental and natural conditions, the pandemic virus remains transmissible. Thus, the pandemic in humans may be accompanied by a panzootic in swine (Peiris et al. 2009b).

Continued circulation of the pandemic (H1N1) 2009 virus in regions where avian H9N2 or HPAI H5N1 viruses continue to be endemic presents opportunities for reassortment. Alarmingly, porcine infections with HPAI H5N1 viruses have been relatively asymptomatic. Previous studies of HPAI H5N1 viruses indicate that pigs have low susceptibility and cannot support transmission of these viruses (Lipatov et al. 2008; Choi et al. 2004c). Such experimental results appear to be also occurring in field isolates (Takano et al. 2009; Li et al. 2004). Repeated subclinical infections could allow undisrupted adaptation and genetic evolution of influenza viruses, favoring untraceable genesis of more virulent strains that are capable of causing pandemics. Then, the mild nature of epidemiology and infectivity might be dramatically changed by genetic recombination with the current pandemic virus. Peiris et al. (2009b) argued that such events might be possible but were not particularly likely. Human-like H3N2 viruses, which have been endemic in swine populations in China, have failed to swap genes with the H5N1 panzootic in poultry and produce a novel reassortant virus of public health concern (Peiris

et al. 2009b). The current novel pandemic virus possesses the specific TRIG segment complex, which enhanced the fitness of North American SIVs to spread rapidly through swine populations and provided a high propensity to reassort (Webby et al. 2000; Brockwell-Staats et al. 2009). Thus, production of a novel reassortant virus between notable avian viruses (i.e., H5, H9, and H7) and a highly transmissible SIV could be, for the first time, a possibility. Such reassortment would be the worst-case scenario or as Peiris et al. (2009b) aptly put it, a nightmare scenario. Turkeys might also provide an alternative host to unify these viruses and facilitate reassortment.

6 Concluding Remarks

The genetic makeup of influenza A viruses currently circulating among pigs throughout Asia is not as well known as that of the widely studied North American and European viruses. Although several SIV subtypes are uniquely found in Asian pigs, European and North American lineage SIVs have already infiltrated and evolved among swine populations in Asia. Thus, pigs in Asia could provide sites for genetic convergence and reassortment of the different lineages of SIVs. In addition, if frequent interspecies transmission to pigs continues in Asia, then novel influenza viruses with significant zoonotic potential could emerge.

The occurrence of the swine-origin pandemic (H1N1) 2009 virus in humans is a public reminder of the significant role of pigs in the genesis, evolution, and perpetuation of influenza A viruses with global public health importance. It also demonstrates that none of the circulating influenza virus strains should be neglected as potential causes of human epidemics and pandemics. Certainly, preventing new influenza outbreaks is practically impossible. However, influenza virus surveillance in humans and animals could play major roles in the event of a looming outbreak or pandemic threat. Details gathered using such an approach would be essential to the research community and enable health authorities to strategically plan and quickly implement appropriate response measures. Therefore, routine epidemiosurveillance of influenza virus infection in pig populations is a thrust of great importance.

Acknowledgments This work was supported through a grant (Grant No.: A103001) from the Korea Healthcare Technology R&D Project by the Ministry of Health & Welfare, Republic of Korea.

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Swine Influenza Virus Vaccines: To Change or Not to Change—That's the Question

Kristien Van Reeth and Wenjun Ma

Abstract Commercial vaccines currently available against swine influenza virus (SIV) are inactivated, adjuvanted, whole virus vaccines, based on H1N1 and/or H3N2 and/or H1N2 SIVs. In keeping with the antigenic and genetic differences between SIVs circulating in Europe and the US, the vaccines for each region are produced locally and contain different strains. Even within a continent, there is no standardization of vaccine strains, and the antigen mass and adjuvants can also differ between different commercial products. Recombinant protein vaccines against SIV, vector, and DNA vaccines, and vaccines attenuated by reverse genetics have been tested in experimental studies, but they have not yet reached the market. In this review, we aim to present a critical analysis of the performance of commercial inactivated and novel generation SIV vaccines in experimental vaccination challenge studies in pigs. We pay special attention to the differences between commercial SIV vaccines and vaccination attitudes in Europe and in North America, to the issue of vaccine strain selection and changes, and to the potential advantages of novel generation vaccines over the traditional killed SIV vaccines.

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Current Topics in Microbiology and Immunology (2013) 370: 173–200
DOI: 10.1007/82_2012_266

Published Online: 13 September 2012

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1 Introduction

Swine influenza, an infection with type A influenza viruses of subtype H1N1, H3N2, or H1N2, is a major cause of acute respiratory disease outbreaks in pigs. The infection is rarely fatal and recovery can be as sudden as the onset of disease. Yet finishing pigs may show temporary weight loss or growth arrest, which cause economic losses, and sows may show reproductive failure due to the high fever. Swine influenza viruses (SIVs) may also contribute to more chronic and multifactorial respiratory disease problems, the so-called porcine respiratory disease complex, in concert with agents like porcine reproductive and respiratory syndrome virus and *Mycoplasma hyopneumoniae*. At the same time, SIVs are enzootic in swine-dense regions worldwide and many if not most infections are very mild or subclinical (reviewed in Van Reeth et al. 2011). The cost of vaccination can thus exceed the benefit gained, and the demand for vaccines is low in some regions.

All current commercial SIV vaccines are traditional inactivated vaccines for intramuscular (IM) injection. The production methods and immunological basis of protection resemble those of inactivated influenza vaccines for humans and horses. But there are also marked differences. One such difference is the lack of a formal system for recommending SIV vaccine strains. Vaccine strain selection is more complicated now than in the past, because several novel SIV subtypes and lineages have emerged during the last 10–15 years. Also, the prevailing SIV strains vary widely in different geographic regions. Several types of novel generation vaccines

for SIV have been developed and tested experimentally, but none are ready for commercial use.

Although inactivated SIV vaccines have been used for decades, detailed information about their composition or critical analyses of their efficacy are hard to find. Published vaccination challenge studies with any type of SIV vaccine should be interpreted with caution, because of multiple differences in their experimental designs and methodology, as well as differences with the field situation. This chapter is intended as a critical review of the performance of traditional and novel generation vaccines for SIV in experimental studies and a thoughtful analysis of their strengths and weaknesses in the field. We focus on the commercial, inactivated SIV vaccines, which were discussed only briefly in previous reviews (Ma and Richt 2010; Thacker and Janke 2008; Vincent et al. 2008b). We will pay special attention to the differences between the European and North American situation, as well as to the issue of vaccine strain selection. This chapter starts with a recapitulation of the pathogenesis and immune response to SIV, which will help to understand the strengths and limitations of various types of influenza vaccines.

2 Principles of Pathogenesis and Immune Response

2.1 Pathogenesis of SIV

The pathogenesis of influenza in pigs is very similar to that in humans, which makes pigs a valuable model to study influenza pathogenesis in a natural host (De Vleeschauwer et al. 2009; Khatri et al. 2010; Van Reeth et al. 1998). SIV replication is limited to epithelial cells of the upper and lower respiratory tract of pigs—the nasal mucosa, ethmoid, tonsils, trachea, and lungs—and virus excretion and transmission occur exclusively via the respiratory route. Infectious virus can thus be isolated from the tissues mentioned, as well as from tonsils, bronchoalveolar lavage (BAL) fluid, and nasal, tonsillar, or oropharyngeal swabs (Brown et al. 1993; De Vleeschauwer et al. 2009; Heinen et al. 2001b; Khatri et al. 2010; Landolt et al. 2003; Richt et al. 2003). In most experimental studies, the virus can be isolated from day 1 post inoculation (PI) onwards and becomes undetectable after day 7. SIV has a preference for the lungs over the upper respiratory tract (De Vleeschauwer et al. 2009; Khatri et al. 2010). The virus is unlikely to spread beyond the respiratory tract and there is generally no detectable viremia.

Infection with SIV can be easily reproduced by experimental inoculation of influenza naïve pigs via the intranasal (IN), aerosol, or intratracheal (IT) route. However, the kinetics of virus replication and the viral loads in various parts of the respiratory tract are markedly dependent upon the inoculation route and dose, and so are the severity of lung inflammation and disease. To illustrate, virus can be recovered from the nasal mucosa and nasal swabs from the first day after IN inoculation, but only 2–3 days after IT inoculation and at lower titers

(De Vleeschauwer et al. 2009). Lung virus titers, in contrast, peak more rapidly and are generally higher after IT inoculation. Within 24 h after IT inoculation of a high virus dose (7.0–7.5 $\log_{10}$ EID50), virus titers may exceed 10^8 infectious particles per gram lung tissue. Only this method results in the characteristic infiltration of the lungs with neutrophils and the typical and abrupt swine flu symptoms—tachypnea and dyspnea with a forced abdominal respiration, fever exceeding 41 °C, dullness, and loss of appetite (De Vleeschauwer et al. 2009; Haesebrouck et al. 1985; Van Reeth et al. 1998, 2002). Less intensive methods—IN inoculation, or IT inoculation of a lower virus dose—lead to a slower buildup of the viral load in the lungs, milder lung inflammation, and a subclinical infection or less specific symptoms: nasal discharge, sneezing, a low to moderate fever (Brown et al. 1993; Larsen et al. 2000; Richt et al. 2003). The viral load in the lungs is proportional to the titers of interferon-α and -γ, tumor necrosis factor-α, and the interleukins-1, -6, and -12 in BAL fluids. These cytokines reach much higher levels after an intense IT than after an IN inoculation, and this appears to determine the difference between subclinical infection and disease. Effective SIV vaccines can greatly reduce or prevent virus replication in the lungs upon IT challenge, and this was found to be associated with reduced cytokine titers in BAL fluids and clinical protection (Van Reeth et al. 2002). Grossly visible lung consolidation has been observed with any inoculation method. The percent of affected lung tissue varies greatly within and between experimental studies (Khatri et al. 2010; Landolt et al. 2003; Richt et al. 2003) and it is no reliable correlate of lung virus titers or disease severity. There is no convincing evidence for differences in pathogenesis or virulence between SIV lineages or strains. The differences reported in some experimental studies seem to be due to biological variation between pigs, experimental variation, or differences in replication competency between viruses (Landolt et al. 2003; Richt et al. 2003; Vincent et al. 2006, 2009).

2.2 The Immune Response After Infection with Live SIV

Our general knowledge of the immune response to influenza virus mainly comes from experiments in mice, which are not natural influenza virus hosts, and from the analysis of prototype human H1N1 and H3N2 strains. The reader is directed to other texts for a more comprehensive overview on influenza virus immunology (Cox et al. 2004; Dormitzer et al. 2011; Wright et al. 2007). Here, we will only recapitulate the basics. Antibody responses mainly develop to the hemagglutinin (HA), neuraminidase (NA), matrix (M), and nucleoprotein (NP). However, only antibodies to the most variable viral protein, HA, can block attachment of the virus to host cell receptors and thus neutralize viral infectivity. These antibodies can be measured in hemagglutination inhibition (HI) or virus neutralization (VN) assays. NA antibodies can inhibit the enzymatic activity of this protein and prevent the release of newly formed virus particles from infected cells. Antibodies to the NP and M can contribute to the killing of infected cells by antibody-dependent

mechanisms, but they cannot prevent an infection. T cells are more broadly directed against conserved regions in all internal and surface proteins of the virus. CD4 + or helper T cells are categorized into T helper 1 and T helper 2 cells, which help B cells and CD8 + T cells, respectively, to proliferate and differentiate into antibody-producing plasma cells and cytotoxic T lymphocytes (CTLs). CTLs are the key players of the cell-mediated immune (CMI) response, because they can kill virus-infected cells directly and thus contribute to virus clearance from the lungs and recovery. The CTL response is directed mainly toward epitopes on the NP, which is relatively conserved among influenza A viruses (Bui et al. 2007). To be recognized by T cells, viral antigens must be degraded into small immunogenic peptides in antigen-presenting cells and presented on the surface of these cells in association with molecules of the major histocompatibility complex (MHC). CD4 + T cells are activated by peptides derived from exogenous, phagocytosed antigens, which are displayed by MHC class II molecules. In contrast, CD8 + T cells are activated by peptides that are generally derived from de novo, intracellular synthesis of viral protein and these are presented in association with MHC class I molecules.

The immune response after infection with live influenza virus is initiated in the lymphoid tissue underlying the mucosae of the airways. Dendritic cells and macrophages rapidly exit the respiratory tract. They travel via afferent lymph to regional lymph nodes or via the blood to the spleen, to deliver antigen to naïve T and B cells. All T cell subpopulations are stimulated, and T cells and antibody-producing B cells will not only reach the circulation, but also the mucosae of the airways and primary and secondary lymphoid organs. The response is very rapid and efficient, and the virus is cleared in about a week. Antibody titers and immune cells wane over time, but populations of memory T and B cells are maintained in the airways and in lymphoid tissues. These cells will mount an amplified and accelerated response after a second encounter with influenza virus.

Our specific knowledge of the immune response to influenza viruses in the pig is patchy. Most studies have focused on the antibody response in serum, while mucosal antibodies in the respiratory tract are likely most important for protection. HI and VN antibodies can be detected in serum by 7–10 days PI and peak by 2–3 weeks PI (Heinen et al. 2000; Larsen et al. 2000; Van Reeth et al. 2006). Post-infection pig sera also contain antibodies to the NA and NP (Heinen et al. 2000, 2001a; Van Reeth et al. 2003a), moderate antibody levels to M1, and low and variable antibody levels to the external part of M2 (Kitikoon et al. 2008). Antibodies to whole virus or to the viral NP have also been found in nasal and lung lavage fluids (Heinen et al. 2000, 2001a; Kitikoon et al. 2006, 2009; Larsen et al. 2000, 2001). As would be expected, serum antibody was predominantly IgM and later IgG, whereas IgA was the main isotype in nasal washes (Heinen et al. 2000; Larsen et al. 2000, 2001). Larsen et al. (2000, 2001) could demonstrate IgA antibody-secreting cells in nasal mucosal tissue, which proves that IgA antibodies are locally secreted. Based on these data and studies in other species, it is believed that secretory IgA is the principal mediator of nasal immunity, whereas IgG transudated from serum contributes more to immunity in the lung. However,

substantial IgA levels have also been found in BAL fluids of SIV-infected pigs (Kitikoon et al. 2006, 2009; Larsen et al. 2000) and in lung lysates (Khatri et al. 2010), suggesting that antibodies may also be locally produced in the lung parenchyma. Also, moderate numbers of cells producing IgG have been demonstrated in the pig's nasal mucosa, next to more numerous IgA secreting cells (Larsen et al. 2000). Data on CMI in the pig are even more limited, and studies have mainly measured T helper cell activity in the circulation, because it is technically difficult to quantify CTLs in outbred animal species or to demonstrate T cells in the airways. Virus-specific lymphoproliferation of peripheral blood mononuclear cells was increased from 7 days PI onwards (Heinen et al. 2001a, b; Van Reeth et al. 2009). These cells show cross-reactive specificities in in vitro cultures: they also react with antigenic variants of the virus to which the animals were exposed, and to a lower extent with influenza A viruses of other subtypes. Cells secreting IFN-γ in response to in vitro restimulation with virus, which are a measure of virus-specific CD4 + and/or CD8 + T cell responses, have been demonstrated in the tracheobronchial lymph nodes and spleen (Khatri et al. 2010; Larsen et al. 2000).

After a primary infection with SIV, there is a solid and long-lasting protection against reinfection with the same or a similar virus strain. HA-specific, neutralizing antibodies in serum, and even more so at the mucosae are thought to be the main mediators of such "homologous" immunity. Under experimental conditions, pigs may be at least partially protected against genetically diverse viruses of the same HA subtype (heterovariant protection) and, in a few cases, against viruses of another HA subtype (heterosubtypic protection). This finding is important, because of the increasing number of novel subtypes and genetic variants isolated from pigs (summarized in Table 1). Importantly, this broad protection appears to be independent of cross-reactive HI antibodies in serum. As an example, pigs that had been previously infected with the earliest H1N1 SIV dating back to 1930 (α-clade) were completely protected against challenge infection with a 2003 North American H1N2 SIV (γ-clade) 5 weeks later, despite up to 21 % nucleotide sequence difference in the HA1 region of the viruses (Vincent et al. 2008a). In similar experiments, a prior infection with European avian-like H1N1 SIV protected pigs against challenge with a North American H1N1 SIV (De Vleeschauwer et al. 2011) or with the 2009 pandemic H1N1 (pH1N1) virus (Busquets et al. 2010). In all three studies, nasal excretion of the challenge virus was undetectable in most pigs. Weaker cross-protection has been observed between H1 SIVs with greater phylogenetic divergence, such as the European H1N1 and H1N2 SIVs, or between H1N1 and H3N2 (Heinen et al. 2001a; Van Reeth et al. 2003a). The European H1N2 virus still caused an infection of H1N1 infection-immune pigs, but it was shed during only 4–5 days, compared to 6 days in influenza naive pigs. However, the extent of cross-protection against H1N2 was dramatically enhanced in pigs that had been infected with both the antigenically distinct H1N1 subtype and H3N2 (Van Reeth et al. 2003a). Despite the absence of cross-reactive serum HI antibodies between the viruses used in all these studies, some studies showed cross-reactive serum antibodies in VN or neuraminidase inhibition (NI) assays, as well

Table 1 Major SIV lineages in Europe and North America

Continent	Year of introduction	Subtype/ lineage	Origin of genes	Comments
Europe	1979	Avian-like H1N1	All eight genes avian-like	
	1984	H3N2	Human HA and NA (A/Hong Kong/68), internal genes from avian-like H1N1 SIV	Wholly human A/Hong Kong/68-like viruses reported in swine since 1970s, reassortment with avian-like H1N1 circa 1984
	1994	H1N2	Human HA (A/Chile/1/83), other genes from H3N2 SIV	
	2009	Pandemic 2009 H1N1	See below	
North America	1918	Classical H1N1 (cH1N1, α-clade)	1918 pandemic H1N1 virus	Virus likely circulating in pigs since human 1918 H1N1 pandemic, first isolated from pigs in 1930
	1998	Triple reassortant (tr) H3N2	HA, NA, and PB1 of human virus (A/Sydney/97) origin; M, NP, and NS of classical swine virus origin; PA and PB2 of North American avian virus origin	Now four phylogenetic clades: I–IV
	2000–2002	Reassortant H1N1 and H1N2 (β- and γ-clade)	HA and NA from cH1N1 SIV (H1N1), triple reassortant internal gene (TRIG) constellation from tr H3N2 SIV; or HA from cH1N1 SIV, NA, and other genes from tr H3N2 SIV (H1N2)	β- and γ-clade are distinct genetic/antigenic clusters of the HA of cH1N1
	2003–2005	Human-like H1N1 and H1N2 (δ-clade)	HA and/or NA from human seasonal H1N1 or H1N2, other genes from trH3N2	Two subclades: δ1, δ2
	2009	Pandemic 2009 H1N1	NA and M from European avian-like H1N1 SIV, other genes from γ-clade H1N2 SIV	

as antibodies in nasal or BAL fluids and/or CMI responses, but their relative contribution to the broad protection remains to be examined. T cell-based immune responses are obviously more cross-reactive than antibody to the HA or NA and, according to older investigations in humans, mucosal IgA is also more cross-reactive than serum IgG (Shvartsman et al. 1977; Waldman et al. 1970). Both immune mechanisms are considered prime mediators of heterosubtypic immunity in the mouse model of influenza (reviewed in Grebe et al. 2008). Yet the experiments in mice also point toward a combined role of multiple cellular and humoral immune components, and they have often yielded confusing and conflicting results. In addition, cross-protection between influenza viruses appears to be much more robust in mice than in natural influenza virus hosts, and it may be less pronounced in the field than under experimental conditions.

2.3 *The Immune Response After Vaccination with Killed SIV Vaccine*

The commercial inactivated SIV vaccines are administered by deep IM injection into the neck. Antibody is presented to cells of the immune system in the draining lymph nodes, where B cells and CD4 + T cells are stimulated. The principle underlying killed influenza virus vaccines in general is the induction of serum antibody to the viral HA. In theory, the vaccines should also induce antibodies to the NA, but the NA antibody response to human influenza vaccines appears to be inconsistent (Dormitzer et al. 2011) and there are no data for SIV vaccines. The antibodies are passively transferred to the mucosae of the respiratory tract by transudation, where they can contact and neutralize influenza virus. There are two major weaknesses of the immune response induced by killed virus vaccines. First, such vaccines induce only serum antibodies, no mucosal antibodies. In comparative experiments, IgA in respiratory secretions and IgA-producing cells in the nasal mucosa were found in pigs infected with live SIV but not in pigs immunized with killed vaccines (Heinen et al. 2001b; Larsen et al. 2001). In addition, vaccine-induced serum HI antibody titers decline rapidly between 2 and 6 weeks after the booster vaccination (Kyriakis et al. 2010a). Second, inactivated vaccines in general do not enter the endogenous pathway of antigen presentation and are unable to activate virus-specific CD8 + T cells or a CTL response. Pigs vaccinated with commercial SIV vaccines may show substantial virus-specific lymphoproliferation responses of PBMC (Heinen et al. 2001b), and similar numbers of IFN-γ secreting lymphocytes in the spleen as compared to infected pigs (Larsen et al. 2001). However, these assays probably largely measure T-helper cell activity, and only the pigs infected with live SIV had IFN-γ secreting cells in the airway mucosa.

The process of transudation of serum IgG is supposed to be more efficient in the lung than in the nasal mucosa (reviewed in Graham and Crowe 2007). Consistent with this is the general notion that killed SIV vaccines mainly reduce pulmonary

virus replication and disease in the vaccinee, whereas reduction of virus replication in the upper respiratory tract and prevention of virus transmission are more difficult to achieve. On the other hand, there are no direct comparative evaluations of antibody levels in various parts of the respiratory tract after influenza vaccination of pigs or other hosts, and their correlation with virus replication after challenge. Such studies would be required to confirm the above assumption and to understand fully how the vaccines work. In pigs, post-vaccination HI antibody titers in the serum of an individual animal do correlate with the reduction in lung virus titers upon IT challenge, provided that HI tests are performed against the challenge strain (Haesebrouck and Pensaert 1986; Kyriakis et al. 2010a; Van Reeth et al. 2001a, b). High HI titers could completely block virus infection of the lungs, while lower HI titers reduced lung virus replication sufficiently to prevent the typical symptoms, which are highly dependent on the viral load in the lungs. In other vaccination challenge studies using various challenge methods, nasal virus excretion was also reduced (Kitikoon et al. 2006; Lee et al. 2007; Macklin et al. 1998) or blocked (Larsen et al. 2001; Kitikoon et al. 2009). As mentioned, we do not know whether the effects on nasal shedding are due to reduced virus replication in the deeper airways or in the nasal mucosa, or to a combination of both. Virus transmission to or from vaccinated pigs is not traditionally assessed, but recent experiments showed a significant reduction of transmission from unvaccinated, challenged pigs to pigs vaccinated with a commercial SIV vaccine (Romagosa et al. 2010).

It is remarkable that serum HI and VN antibody titers are generally greater after a double vaccination of SIV naïve than after infection (Heinen et al. 2001b; Larsen et al. 2001; Vincent et al. 2008a). Post-vaccination HI antibody titers in swine are usually also several times higher than those reported in humans, which is most likely due to the adjuvant in SIV vaccines. At 2–3 weeks after the booster vaccination, HI antibody titers $\geq$320–640 to the homologous strains in the vaccine are common. Such high homologous antibody titers seem to translate in substantial, though lower, titers to heterologous strains (Heinen et al. 2001b; Van Reeth et al. 2001a, b). In one challenge study with a commercial European SIV vaccine, the mean post-vaccination HI antibody titer (1,152) to the antigenically drifted H3N2 SIV used for challenge was 4-fold lower than the homologous antibody titer (3,840) to the vaccine H3N2 strain, but still 4-fold higher than antibody titers induced by infection with the same challenge virus (Heinen et al. 2001b). It came as no surprise, therefore, that the vaccinated pigs showed a solid protection against the heterologous challenge. Only two out of five vaccinated pigs were positive for the challenge virus in oropharyngeal swabs, at barely detectable levels and for only 2–3 days, whereas all five unvaccinated controls were virus-positive for 4–6 consecutive days post challenge. Still, prior infection with the H3N2 virus afforded a complete and thus better protection than the vaccination, supposedly as a result of a more balanced immune response including mucosal immunity and CTLs. Of paramount importance, however, is that the immune response in pigs in the field is frequently a combination of vaccination and natural infection immunity, as discussed in the next section.

3 Commercial Inactivated SIV Vaccines

Commercial SIV vaccines are traditional, inactivated vaccines produced in eggs or in cell culture. They are administered by deep IM injection into the neck of pigs. Primary vaccination should consist of two injections 2–4 weeks apart. For sow herds, a preferred method is to vaccinate gilts twice, prebreeding, and the sows either quarterly or 3–6 weeks before farrowing. In sow herds with high antibody levels to SIV from either vaccination and/or natural infection, vaccination of piglets should be delayed until the age of 12–16 weeks to avoid interference with maternally derived antibodies (MDA).

SIV vaccines show similarities with the inactivated influenza vaccines for humans, but there are essential differences too. While the human vaccines generally contain purified viral surface antigens without adjuvant (reviewed in Fiore et al. 2009), SIV vaccines are mostly whole virus preparations with an oil-based adjuvant. Unlike human vaccines, SIV vaccines are not standardized for antigenic dose and vaccine strains. There is no formal system for recommending SIV vaccine strains or updates, and strain selection is complicated by several factors. The prevailing SIV strains and their genotypes differ between continents and regions; multiple SIV subtypes and lineages are circulating concurrently within each continent, and several new lineages have emerged during the last 10–15 years. Table 1 summarizes the major SIV lineages in Europe and North America; more detailed information can be found elsewhere in this book.

In keeping with the antigenic and genetic differences between SIVs in Europe and in North America, the vaccines for each geographic region are produced locally and they contain entirely different strains. Within each continent, the vaccine strains may differ among different products, as well as the exact adjuvant formulation and antigen dose. This is illustrated in Table 2 for European vaccines and Table 3 for North American vaccines. Apart from several regulatory issues, the attitude toward vaccination also differs between Europe and North America. Vaccine uptake is higher in the US, where approximately 70 % of breeding stock are estimated to be vaccinated (Vincent et al. 2008b). Updating vaccine strains is considered more important in North America than in Europe. Autogenous vaccines containing herd-specific strains are also very popular in the US, but they are not further discussed here. SIV vaccines are also available in combination with other swine pathogens, such as porcine parvovirus, *Mycoplasma hyopneumoniae*, and, in some countries, Aujeszky's disease virus, to name a few.

3.1 SIV Vaccines in Europe

SIV vaccines are commercially available in most, though not all European countries. Most vaccines were initially licensed during the mid-1980s or early 1990s. They contain the two influenza virus subtypes that were prevalent at that

Table 2 Major commercially available SIV vaccines in Europe in 2011

Manufacturer	Product name	Influenza virus strains	Adjuvant	Antigenic content[d] per vaccine dose
Merial	Gripovac[a]	A/New Jersey/8/76 (H1N1) A/Port Chalmers/1/73 (H3N2)	Oil	H1N1: ≥1.7 HIU H3N2: ≥2.2 HIU
Pfizer Olot	Suvaxyn Flu	Sw/Netherlands/25/80 (H1N1) A/Port Chalmers/1/73 (H3N2)	Oil	H1N1: 4 μg HA H3N2: 4 μg HA
Hipra	Gripork	Sw/Olost/84 (H1N1) A/Port Chalmers/1/73 (H3N2)	Oil	H1N1: 3×10^7 EID_{50} H3N2: 2.5×10^7 EID_{50}
Impfstoffwerk Dessau-Tornau	Respiporc Flu[b]	Sw/Belgium/230/92 (H1N1) Sw/Belgium/220/92 (H3N2)	Aluminum hydroxide—oil	H1N1: ≥256 HAU H3N2: ≥256 HAU
Impfstoffwerk Dessau-Tornau	Respiporc Flu3[b, c]	Sw/Haselunne/2617/03 (H1N1) Sw/Bakum/1769/03 (H3N2) Sw/Bakum/1832/00 (H1N2)	Carbomer	H1N1: $\geq 10^7$ $TCID_{50}$ H3N2: $\geq 10^7$ $TCID_{50}$ H1N2: $\geq 10^7$ $TCID_{50}$

[a] Split vaccine, other vaccines contain whole virus; production stopped in 2010
[b] Produced in cell culture, other vaccines are produced in eggs
[c] The vaccine is marketed by Merial under the trademark of Gripovac 3
[d] HIU: hemagglutination inhibiting units as determined by measuring the HI antibody response after administration of the vaccine to pigs; HAU: hemagglutinating units before inactivation as determined in a hemagglutination assay with chicken red blood cells; $TCID_{50}$: Tissue culture infectious dose 50 % before inactivation; EID_{50}: egg infectious dose 50 % before inactivation

time, H1N1 and H3N2, and the vaccine strains have never been updated (Table 2). H1N2 SIVs with a human-like H1 have become widespread in swine in Europe since the mid-late 1990s, but a trivalent vaccine including H1N2 was only licensed in 2010. As shown in Table 2, one cannot compare the antigenic mass of various commercial products, because different manufacturers use different methods to measure and express the amount of antigen. The 2009 pH1N1 influenza virus has been reported from pigs in several European countries, but European experts and authorities believe that there is no need for a specific pH1N1 vaccine at this time.

Licensing requirements for SIV vaccines in Europe include a stringent efficacy test for each influenza subtype in the vaccine, which is described in the European Pharmacopoeia. Influenza virus-seronegative pigs must be vaccinated twice according to label directions and challenged with a field isolate of SIV by the IT route. Half of the pigs must be euthanized at 24 h after the challenge, the other half at 72 h. The vaccine complies with the test if mean virus titers in the lungs of vaccinated pigs are significantly lower than in unvaccinated controls at both time

points. The requirement to demonstrate a beneficial effect of vaccination on fever and weight loss has been omitted from the revised European Pharmacopoeia monograph in 2003, because of the difficulty to reproduce the typical flu symptoms by experimental inoculation and the significant correlation between lung virus titers and disease. Manufacturers also have to execute the full licensing procedure if they simply want to change vaccine strains. This is part of the reason why most European SIV vaccines have remained with outdated strains.

Most published SIV vaccine efficacy data are from experimental vaccination challenge studies in which SIV-seronegative pigs are vaccinated twice with commercial vaccine and challenged with field isolates of SIV 2–6 weeks after the second vaccination. Because of the European Pharmacopoeia requirements, studies with European SIV vaccines mainly use IT challenge. The pigs are usually euthanized during the very acute stage of infection, 1 or 3 days after challenge. Lung virus titers are the main criterion to evaluate protection. Most studies have been performed with the first commercial vaccine against SIV, which is no longer produced since 2010 (see Table 2). The human A/New Jersey/8/76 strain in this vaccine belongs to the classical H1N1 virus lineage and is more closely related to SIVs in North America than to the European avian-like H1N1 SIV lineage. The A/Port Chalmers/1/73 strain, which is also included in other European SIV vaccines, is the supposed human precursor virus of the European swine H3N2 lineage. In initial studies the vaccinated pigs were challenged IT with a very high dose (7.5 $\log_{10}$ EID50) of H1N1 and H3N2 SIVs isolated in Belgium in 1983–1984 or later in 1998 (Vandeputte et al. 1986; Haesebrouck and Pensaert 1986; Van Reeth et al. 2001a, b). Under these conditions, unvaccinated challenge control pigs invariably demonstrate high virus titers in the lungs, up to 7.0–8.0 $\log_{10}$ ID50. They also show severe though transient dyspnea, fever up to 41 °C, depression, and anorexia that are so typical of acute outbreaks of SIV. The vaccine offered excellent virological protection against this severe virus challenge, and the lungs of about 50 % of the vaccinated pigs tested negative for the challenge virus. The remaining pigs had reduced lung virus titers, and all pigs were protected against disease. The same vaccine also offered significant protection against aerosol challenge with a 1996 H3N2 SIV from the Netherlands, as shown by reduced virus isolation rates in oropharyngeal swabs (Heinen et al. 2001b). More recently, this vaccine and three other commercial vaccines were compared for their efficacy against challenge with a Belgian H1N1 SIV isolated in 2007 (Kyriakis et al. 2010a). The pigs were inoculated IT with a moderate virus dose (5.0 $\log_{10}$ EID50), which does not evoke typical SIV symptoms, but still results in reproducibly high virus titers in the lungs of unvaccinated control pigs. Although all vaccines reduced lung virus titers at days 1 and 3 post challenge, the reduction was significant for only two of the four vaccines (Kyriakis et al. 2010a).

Table 3 Major commercially available SIV vaccines in North America in 2011

Manufacturer	Product name	Influenza virus strains[c]	Adjuvant[d]
Novartis	PneumoStar[a] SIV	α-cluster H1N1 cluster I H3N2	Immunstar®
Intervet/Schering-Plough Animal Health	MaxiVac Excell[b] 3.0	α-cluster H1N1 β-cluster rH1N1 cluster I H3N2	Emunade®
Intervet/Schering-Plough Animal Health	MaxiVac Excell 5.0	β-cluster H1N1 γ-cluster H1N1 δ-cluster H1N1 cluster I H3N2 cluster IV H3N2	Emunade®
Pfizer Animal Health	FluSure Legacy	α-cluster H1N1 cluster I H3N2	Amphigen®
Pfizer Animal Health	FluSure XP	γ-cluster H1N1 δ-cluster H1N1 cluster IV H3N2	Amphigen®
Pfizer Animal Health	FluSure Pandemic	Pandemic 2009 H1N1	Amphigen®
Pfizer Animal Health	FluSure XP	γ-cluster H1N1 δ2-cluster H1N1 cluster IV H3N2 δ1-cluster H1N2	Amphigen®

[a] Pneumostar is the only single-dose SIV vaccine
[b] MaxiVac Excell is a registered trademark of Intervet/Schering-Plough Animal Health
[c] Exact strain names and antigen dose are proprietary for most vaccines
[d] All adjuvants are oil-in-water emulsions, except for Immunstar®, which is water-in-oil-in-water

3.2 SIV Vaccines in North America

In North America, a monovalent H1N1 SIV vaccine based on classical H1N1 virus became first available in 1994. Responding to the changing SIV epidemiological situation, manufacturers have reformulated their vaccines in recent years. After the emergence of H3N2 influenza viruses in the US swine population in 1998, monovalent H3N2 and ultimately multivalent H1/H3 SIV vaccines were launched. A monovalent vaccine based on the 2009 pH1N1 virus was licensed in 2009. Of the seven vaccines listed in Table 3, four contain SIVs of multiple H1 and/or H3 clusters.

The licensing requirements for SIV vaccines in the US are different from those in Europe. In September 2007, the United States Department of Agriculture introduced new licensing guidelines for updating strains in currently licensed vaccines. Since then, immunogenicity of a novel strain can be demonstrated by serology in pigs rather than challenge. Manufacturers thus have the opportunity to address vaccine updates in a more timely and responsive manner compared to the hurdles in Europe.

In studies with US SIV vaccines, pigs are often challenged as early as 10–14 days after the second vaccination. Challenge is performed via the IN route or IT with a moderate virus dose. The pigs are euthanized relatively late in the course of infection, day 5 or later, and virus titers in lung tissue are rarely determined. Instead, investigators evaluate nasal virus shedding, lung lesions, and clinical signs, though it must be said that disease is very mild with the challenge methods used. There are published efficacy studies with the first, monovalent H1N1 vaccine (Macklin et al. 1998, Larsen et al. 2001) and with bivalent H1N1/H3N2 vaccines (Lee et al. 2007, Kitikoon et al. 2006, 2009), but not with the most recent multivalent vaccines. The vaccines were shown to reduce clinical scores and macroscopic and microscopic lung lesions, and nasal virus excretion was either reduced (Kitikoon et al. 2006; Lee et al. 2007; Macklin et al. 1998) or undetectable (Kitikoon et al. 2009; Larsen et al. 2001). Interestingly, two studies with the monovalent H1N1 vaccine and IN challenge with the same classical H1N1 SIV from 1988 yielded discrepant results. Nasal shedding of the challenge virus was undetectable in one study (Larsen et al. 2001), but reduced by only 1–2 $\log_{10}$ in the other (Macklin et al. 1998). This difference may be due to the 10-fold lower virus challenge dose used in the former study, and it is a good example of the huge impact of small variations in the experimental design on the outcome of such studies.

3.3 Implications of Antigenic Variation for Vaccine Efficacy

Over time, SIVs undergo antigenic drift in their HA, though at a slower pace than human influenza viruses (de Jong et al. 2007). Another, unique characteristic of SIVs is that multiple genetically diverse lineages of H1 and H3 viruses may coexist (reviewed in Van Reeth et al. 2011; Vincent et al. 2008b). The sequences of the HAs of some H1 SIV lineages at the amino acid (aa) level differ by as much as 20–25 %. Such large genetic differences exist between the three H1 lineages in Europe—avian-like H1N1, H1N2, and 2009 pH1N1—and between North American H1 SIVs of clade δ versus the other clades. Smaller genetic differences occur between the α, β, and γ H1 clades, and between North American H3 clades, as well as within each of the virus lineages. Unfortunately, we do not know exactly how much drift or genetic diversity is necessary to require substitution or addition of vaccine strains. In addition, the location of the changes on the HA protein may be more important than the total amount of change.

Several commercial vaccines in Europe have clearly shown the ability to provide protection against H1N1 and H3N2 SIVs isolated over many years and with considerable antigenic and genetic drift compared to the vaccine strains. Independent studies with Port Chalmers/73-based vaccines showed significant virological protection against challenge with H3N2 SIVs isolated in 1984, 1996, 1998, and 2008 (Heinen et al. 2001b; Van Reeth et al. 2001a, and unpublished). The challenge viruses were 84–92 % similar to the Port Chalmers strain in the aa sequence of their HA1. It is also striking that a classical H1N1 (New Jersey/76)-based vaccine could

protect against poorly related avian-like H1N1 SIVs from the 1980s and 1990s, with only 78–81 % aa homology to the vaccine strain (Haesebrouck and Pensaert 1986; Van Reeth et al. 2001b). However, the same vaccine did not protect against a 2007 H1N1 SIV with only slightly lower homology (77 %). Protection against other H1 lineages was somewhat inconsistent with the bivalent H1N1/H3N2 vaccines. The vaccines failed to induce cross-reactive HI antibodies against the European H1N2 SIV and there was no adequate protection against H1N2 challenge in a study with one of these vaccines (Van Reeth et al. 2003b). These findings were in line with the low genetic homology between the H1N2 virus and the H1N1 vaccine strains (<73 % aa homology in the HA1) and they have led to the development of a trivalent vaccine including H1N2. In serologic investigations with the 2009 pH1N1 virus, two of the four vaccines examined induced HI antibody titers ≥20 to the pandemic virus, even though there was only 72–75 % aa homology between the pandemic H1 and most European H1 vaccine strains (Kyriakis et al. 2010b). In another study, most vaccines induced cross-reactive VN antibodies and partial cross-protection against 2009 pH1N1 challenge (Dürrwald et al. 2010). It must be said though that antibody titers varied strongly between individual pigs, and a specific monovalent vaccine based on the pandemic virus stimulated much higher antibody titers and offered superior protection.

The interpretation of studies with commercial North American SIV vaccines is more difficult, because the vaccine strains are proprietary. In one study, three different commercial SIV vaccines containing genetic cluster I H3N2 viruses offered partial protection against challenge with a cluster III H3N2 virus (Lee et al. 2007). Pigs vaccinated with an experimental homologous vaccine, on the other hand, showed complete sterilizing immunity. Although the adjuvants and antigen mass were also not standardized between the vaccines, the better protection was most likely due to the virus strain. Kitikoon et al. (2006, 2009) have used two different bivalent vaccines containing a classical H1N1 SIV and challenge with a heterologous classical virus. Both studies showed significant protection and the authors concluded that "a complete match between vaccine strains and field strains may not always be required". There are no published studies with commercial vaccines and challenge with H1 viruses from other phylogenetic clades than the vaccine strains. An experimental vaccine based on the classical sw/Iowa/30 strain (α-clade) did not offer complete cross-protection against challenge with a triple reassortant H1N2 SIV, sw/Minnesota/03 (γ-clade) (Kitikoon et al. 2010; Vincent et al. 2008a). In both studies, however, the vaccine reduced nasal swab virus titers at one of two time points tested, either 3 or 5 days PI. Also, the historical sw/Iowa/30 vaccine strain may be more distantly related to H1N2 viruses than the α-clade H1N1 strains in the current vaccines. Soon after the emergence of the 2009 pH1N1 virus in the North American swine population, the authorized vaccines were examined for their ability to induce cross-reactive antibodies and protection. Limited serologic cross-reactivity in the HI test was observed with two of the three commercial vaccines tested (Vincent et al. 2010b). These vaccines contained γ-cluster H1 SIV, which is most closely related to the pandemic virus. Both vaccines demonstrated partial protection against 2009 pH1N1 challenge, but antibody titers and protection were

superior with an experimental monovalent 2009 pH1N1 vaccine (Vincent et al. 2010a).

Thus, on the one hand, the vaccines may offer suboptimal or insufficient protection against challenge with SIVs of a distinct H1 or H3 lineage. On the other hand, though, even major differences between vaccine and challenge strains do not necessarily result in complete vaccine breakdown. The relatively broad serologic reactivity and protection as compared to that reported for human or equine influenza vaccines is most likely due to the oil-based adjuvants in the swine vaccines. Oil-based emulsions do not only increase antibody titers, they also expand the cross-reactivity of the antibody response (reviewed in Dormitzer et al. 2011). Several studies with European SIV vaccines point toward an important role of the adjuvant in SIV vaccine potency. One of the best examples is a comparative study with commercial vaccines containing different H1N1 strains and challenge with a 2007 avian-like H1N1 SIV (Kyriakis et al. 2010a). Two oil-adjuvanted vaccines with H1N1 strains showing 93 and 89 % aa homology to the challenge virus offered a solid protection against challenge. In contrast, there was no significant protection with a vaccine with a more closely related H1N1 strain, showing 95 % aa homology to the challenge virus and only three aa changes in known antigenic sites of the HA. The latter vaccine contained a carbomer adjuvant, which is less reactogenic than oil, but also less potent. The very same vaccine also failed to induce cross-reactive antibody titers against the 2009 pH1N1 virus, unlike other European vaccines. However, significant 2009 pH1N1 antibody titers were obtained with an experimental vaccine batch in which carbomer was replaced by mineral oil (Dürrwald et al. 2010). Another study showed complete protection against challenge with a 1998 European H1N1 SIV in pigs vaccinated with the New Jersey/76-based commercial vaccine (Van Reeth et al. 2001b). Pigs vaccinated with an experimental vaccine based on the same virus strain, in contrast, were only partially protected. This discrepancy could only be explained by differences in the adjuvant and/or antigen dose.

3.4 In Practice

Data regarding SIV vaccine efficacy in the field are very limited, and many variables between the experimental situation and the field complicate extrapolation from experimental data. One factor is the time interval between vaccination and exposure to virulent virus, which is frequently longer in nature than in experiments. Perhaps the main difference is that most animals in the field are not influenza naïve. Young piglets may have MDA to SIV until the age of 4–14 weeks and interference of this passive immunity with effective vaccination is a major weakness of killed SIV vaccines (Kitikoon et al. 2006; Loeffen et al. 2003). However, most SIV vaccine is used in gilts and sows, and almost all these animals have been infected with one or more SIVs. Many observations in humans document the important role of priming to potentiate robust subsequent responses to

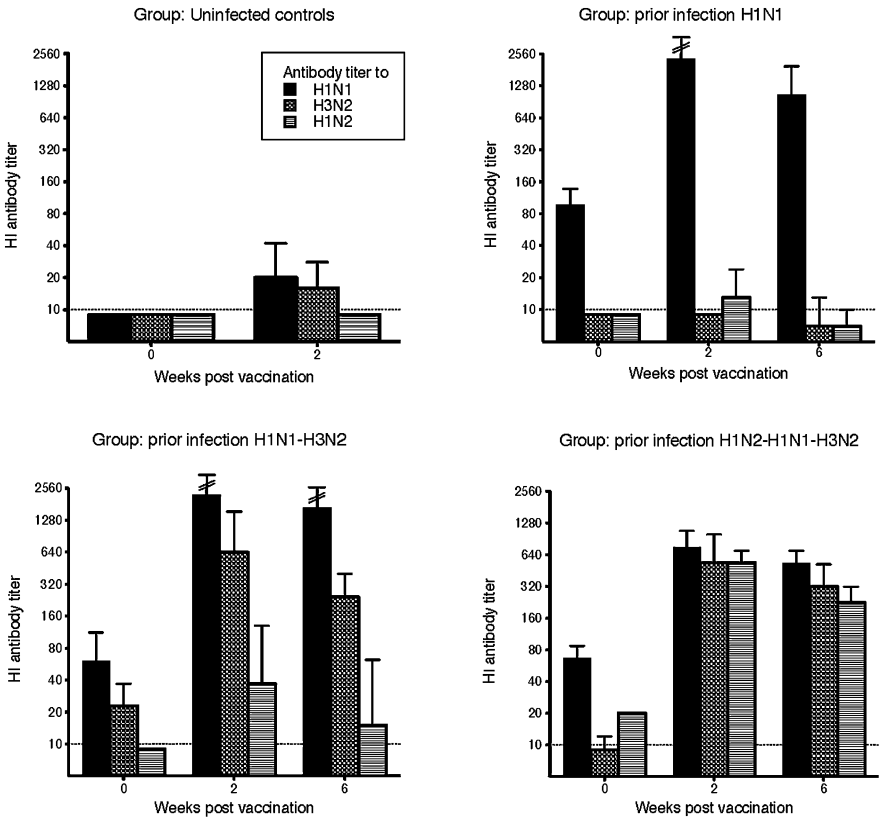

Fig. 1 Comparison of serum HI antibody titers (geometric means and standard deviation) after a single administration of a commercial European SIV vaccine in influenza naive pigs and pigs previously infected with one, two, or three European SIV subtypes. The vaccine was based on A/New Jersey/8/76 (H1N1) and A/Port Chalmers/1/73 (H3N2). Serology was performed against the viruses used for experimental infection of pigs: sw/Belgium/1/98 (H1N1), sw/Flanders/1/98 (H3N2), and sw/Gent/7625/99 (H1N2)

immunization (reviewed in Dormitzer et al. 2011). Priming of the immune system also explains why a single dose of killed influenza vaccine is sufficient to induce protective antibody levels in adults, whereas two vaccine administrations are needed in children. Also in pigs, preexisting immunity appears to have a dramatic impact on the antibody response to killed SIV vaccine as shown in an experimental study (Van Reeth et al. 2006) (Fig. 1). In that study, the pigs were vaccinated only once with a commercial vaccine, a few weeks after they had been inoculated intranasally with live SIV, or with two or three SIVs at 3–6 week intervals. The vaccine H1N1 and H3N2 strains (A/New Jersey/8/76 and A/Port Chalmers/1/73) were only distantly related to the enzootic H1N1, H3N2, and H1N2 SIVs used for infection. In previously influenza naïve pigs, the single vaccination induced minimal HI antibody titers to H1N1 and H3N2 only. In contrast, the

infection-primed pigs showed a dramatic booster of HI antibody titers to any of the viruses they had previously been exposed to, including H1N2. Two weeks after the vaccination of H1N1 infection-immune pigs, for example, mean group antibody titers to H1N1 reached as high as 2,319 in the HI test and 11,230 in the VN test (data not shown). This may mean that a close antigenic match between vaccine and field strains is less important in pigs primed by infection with field strains. Based on findings with avian H5N1 influenza viruses (Sabarth et al. 2010), one could even speculate that booster vaccinations with relatively distant strains may stimulate a more broadly cross-reactive VN antibody response than homologous prime-boost regimes, but this hypothesis remains to be confirmed. The other way round, some researchers conclude that killed SIV vaccines prime pigs for a stronger and more rapid mucosal and cellular immune response after challenge with live virus (Kitikoon et al. 2006, 2009; Vincent et al. 2008a), but other studies showed no differences at all between vaccinated and nonvaccinated pigs (Heinen et al. 2001b).

Taken together, commercial SIV vaccines can be considered reasonably effective in the field for two reasons. First, a minimal reduction of SIV replication is sufficient to protect against the typical, severe symptoms of SIV. Second, many animals are primed by infection with SIV, which will enhance the antibody response to vaccination.

4 Novel Generation Vaccines

The ideal SIV vaccine should induce the broadest immune response possible and overcome interference from MDA, and this has stimulated research into alternative vaccination approaches. To date, several next generation vaccines for SIV have been developed and tested in pigs, but none of them have reached the market. Many of these vaccines have been reviewed extensively in previous articles (Ma and Richt 2010; Thacker and Janke 2008; Vincent et al. 2008b). We will therefore briefly discuss their performance in experimental challenge studies and their potential advantages over the traditional vaccines.

4.1 Recombinant Protein Vaccines

Several studies in mice report protection from a lethal influenza infection mediated by conserved M2 and NP proteins (Jimenez et al. 2007; Neirynck et al. 1999; Wraith et al. 1987). These studies have been interpreted as pointing the way to a universal influenza vaccine. Heinen et al. (2002) have investigated such vaccination strategies in pigs, using a fusion protein between the extracellular domain of the M2 of a human virus and the hepatitis B core protein (M2eHBc), which was protective in mice. They also developed a DNA construct between M2e and NP,

which was expected to induce a T helper and CTL response to the NP in addition to M2e antibodies, and thus superior protection. Pigs were immunized three times via the IM (M2eHBc) or intradermal (DNA) route and challenged by aerosol with an H1N1 SIV from the Netherlands (sw/Best/96) 1 month after the last vaccination. Both vaccines induced the desired immune response, but there was no significant reduction of virus excretion after challenge. Clinical signs were even more severe in the vaccinated pigs than in unvaccinated controls. The authors of this study hypothesize that antibodies to M2e, which are nonneutralizing and kill cells via cell-targeting mechanisms, may induce antibody-dependent cytotoxic hypersensitivity. Kitikoon et al. (2010) obtained a similar poor protection against nasal virus shedding in pigs vaccinated twice with a recombinant M2 protein vaccine and challenged IT with an H1N2 SIV from North America (sw/Minnesota/03). Protection against the heterologous H1N2 challenge increased when the M2 protein was combined with an experimental, inactivated, monovalent H1N1 vaccine (sw/Iowa/30), but it was not better than the protection obtained with the inactivated vaccine alone.

Recently, Loeffen et al. (2011) have constructed a recombinant, soluble trimer of the HA of the prototype pH1N1 virus A/California/04/2009. Such HA trimers are supposed to be better vaccine candidates than HA monomers, because they resemble the natural HA more closely and thus induce higher levels of neutralizing antibodies (Wei et al. 2008). Pigs were vaccinated twice IM with the recombinant and challenged IN with a 2009 pH1N1 virus 3 weeks after the booster vaccination. The pigs developed very high HI and VN antibodies against the homologous virus, which were cross-reactive with a European avian-like H1N1 SIV, but not with H1N2. They were almost completely protected against replication of the challenge virus in the respiratory tract and virus excretion. On the other hand, this study used a high dose of HA and a challenge virus that was closely related to the vaccine strain.

4.2 Vector Vaccines

Expression of influenza virus proteins from viral vectors can safely induce a humoral and cellular immune response comparable to natural infection (Souza et al. 2005), though vector vaccines will likely fail to stimulate significant CTL responses. A human adenovirus serotype 5 (hAd5) vector has been used to express genes of the H3N2 SIV sw/Texas/98. An hAd5 recombinant expressing the HA of this H3N2 SIV has shown partial protection in mice after a challenge with a heterovariant virus, A/Hong Kong/1/68 (H3N2) (Tang et al. 2002). Subsequently, a hAd5 recombinant expressing the NP of sw/Texas/98 (H3N2) was also generated and challenge experiments in pigs were conducted to test the efficacy of both hAd5 recombinant viruses alone and in a mixture (Wesley et al. 2004). Pigs immunized once IM against HA plus NP or against HA alone developed high levels of virus-specific HI antibodies by 4 weeks post vaccination. Upon IN challenge with a closely related H3N2 virus, these pigs showed complete (HA + NP) and nearly complete (HA only)

protection against nasal shedding and lung lesions. In contrast, pigs vaccinated with NP alone did not have HI antibodies at the time of challenge and they did not show much effect on excretion of the challenge virus or lung lesions. Subsequent studies demonstrated that a recombinant hAd5 virus expressing HA + NP was able to prime the immune system in the presence of MDA against SIV, which often interfere with conventional inactivated vaccines (Wesley and Lager 2006). Seven-day-old piglets with MDA against H3N2 SIV were vaccinated once with the vector, boosted with a commercial bivalent vaccine 3 weeks later, and challenged IT with H3N2 SIV 2 weeks after the last vaccination. This resulted in very high post-vaccination HI antibody titers and highly significant protection against challenge. One administration of the vector vaccine alone without the killed vaccine boost was partially protective in pigs with MDA, whereas a single administration of the killed vaccine alone was efficacious in the absence of MDA only. Another study showed that the hAd5 SIV vector was also protective after administration with a needle-free device (Wesley and Lager 2005). Still, it must be taken into account that the pigs in these studies were vaccinated with extremely high doses of the vector (>9.0 $\log_{10}$ TCID50) and that they were challenged with a closely related SIV. Moreover, immunity to the vector virus itself may interfere with booster vaccinations in sows, and with vaccination of their piglets, which are expected to have MDA to the vector.

4.3 DNA Vaccines

DNA vaccines are naked DNA plasmids that have been genetically engineered to produce defined antigens within transfected cells. Intracellular antigens can be presented by MHC class I and II molecules. This will stimulate both humoral and CMI responses, including CTLs. The stable plasmid DNA can be easily produced on a large scale at low costs. DNA vaccines for human and avian influenza viruses have been developed and robust immune responses have been demonstrated in mice, chickens, ferrets, horses, and nonhuman primates following the administration of HA, NP, NA, and M constructs (reviewed in Kim and Jacob 2009; Olsen 2000). There is much less knowledge about the efficacy of DNA-based influenza vaccines in humans (Drape et al. 2006). For SIV DNA vaccine studies, two different DNA vaccine constructs have been used (Eriksson et al. 1998; Macklin et al. 1998). In one study, a double administration of the NP from the human influenza virus A/PR8/34 (H1N1) by gene gun induced a strong antibody response in pigs, but no detectable protection from virus challenge (Macklin et al. 1998). In contrast, pigs immunized with the HA gene from an H1N1 SIV (sw/Indiana/1726/88) showed a minimal reduction of virus shedding after challenge with the homologous virus (Macklin et al. 1998). When pigs were given one dose of a conventional inactivated SIV vaccine 4 weeks after a priming dose of the same HA DNA vaccine, they developed higher serum antibody titers and were protected from challenge to a significantly greater degree than pigs given two doses of DNA vaccine (Larsen et al. 2001). However, two doses of the conventional vaccine were

nearly as effective as the DNA vaccine prime–killed vaccine boost regime. Further studies are needed to determine whether the latter strategy could overcome interference by MDA (Thacker and Janke 2008) or offer a broader protection compared to killed vaccine alone.

Several safety concerns have been raised regarding the use of DNA vaccines. They may integrate into host genomes, increasing the risk of malignancy, and induce autoantibodies against double-stranded DNA, leading to autoimmune disease (reviewed in Kim and Jacob 2009). The need to develop more potent DNA vaccines and more efficient delivery strategies, which allow administration of DNA to easily accessible sites on the pig's body, is a critical challenge for this technology and its clinical use in veterinary medicine.

4.4 Live Attenuated Vaccines

4.4.1 Live Attenuated SIV Vaccine with Modified NS1 Protein

The nonstructural NS1 protein of the influenza A virus is exclusively expressed in virus-infected cells and not present in virus particles. One of the major functions of the NS1 protein of influenza viruses is the inhibition of the type I interferon-mediated antiviral response. Modification of the NS1 can be utilized to produce live attenuated SIVs, which have a great potential as modified live virus (MLV) vaccines. Attenuated SIVs expressing NS1-truncated proteins of an H3N2 SIV (sw/Texas/4199-2/98, Tx/98) with 73, 99, or 126 amino acids (Tx/98 NS1Δ73, Tx/98 NS1Δ99, and Tx/98 NS1Δ126) have been generated using reverse genetics (Solórzano et al. 2005). The Tx/98 NS1Δ126 virus is the most attenuated virus exhibiting the lowest level of NS1 expression and decreased replication in vitro and in vivo when compared to the wild-type virus and both other deletion mutants (Solórzano et al. 2005). IT inoculation of pigs with Tx/98 NS1Δ126 virus induces minimal macroscopic and histopathologic lung lesions. A primary IT vaccination with Tx/98 NS1Δ126 virus followed by a booster IN vaccination 3 weeks later resulted in complete protection against challenge with the homologous Tx/98 virus and partial protection against a heterosubtypic H1N1 virus (Richt et al. 2006). Subsequent studies showed that the IN route was more efficient than the IM route in priming the mucosal antibody response (Vincent et al. 2007). Two doses of Tx/98 NS1Δ126 virus administered IN conferred complete protection against a homologous virus challenge and nearly complete protection against a heterovariant challenge with an antigenically distant H3N2 SIV (sw/Colorado/23619/99). Moreover, IN vaccination slightly reduced virus titers in BAL fluids and nasal swabs at 5 days post challenge of pigs with a heterosubtypic H1N1 SIV (sw/Iowa/00239/2004), though it failed to reduce lung lesions. The absence of serum HI antibodies to the heterovariant and heterosubtypic viruses in this study indicates that a complex host response including both cellular and humoral mechanisms contributes to the broad protection (Vincent et al. 2007), but the immune response

to NS1-modified vaccines needs further study. According to a more recent study, the NS1 vaccine may also be partially effective in piglets with MDA (Vincent et al. 2008b). A major concern with this MLV vaccine, as with all live vaccines, is the possibility of reassortment between field viruses and the vaccine strain, producing novel reassortant viruses. The safety aspects of NS1-modified vaccines are discussed in more detail by Richt and Garcia-Sastre (2009). Concurrent infections with other respiratory pathogens of swine at the time of vaccination could theoretically also enhance lung lesions or illness.

4.4.2 Elastase-Dependent Live Attenuated SIV Vaccine

Stech et al. (2005) demonstrated that the conversion of a conserved cleavage site in the influenza virus HA protein from a trypsin-sensitive motif to an elastase-sensitive motif resulted in attenuation of avian- or mouse-adapted influenza A viruses. Masic et al. (2009a) used the same strategy to generate two elastase-dependent mutant SIVs derived from sw/Saskatchewan/18789/02 (H1N1) called sw/Sk-R345V (R345V) and sw/Sk-R345A (R345A). These two viruses displayed similar growth properties to the wild-type virus in vitro, but were highly attenuated in pigs (Masic et al. 2009a). Administration of either the R345V or R345A via the IT route induced systemic and mucosal antibody responses and CMI. Pigs immunized with the R345V virus had significantly higher HI titers than the R345A-vaccinated animals (Masic et al. 2009b). Therefore, the R345V virus was selected to further test its efficacy against a challenge with homologous and heterologous viruses. A double IT vaccination with R345V provided pigs with complete protection from challenge with the homologous H1N1 virus and the H1N1 variant sw/Indiana/1726/88, and partial protection from heterosubtypic H3N2 infection. It should be noted though that there was tremendous variation in H3N2 virus titers in the lungs between vaccinated pigs. The fact that the vaccination requires two administrations by the IT route is a major disadvantage, because this is not practical for vaccination in the field. The safety of this vaccine also needs to be further investigated.

4.5 In Practice

Novel generation vaccines for SIV have not yet reached the market, because they cannot compete with the existing vaccines for safety, or efficacy, or cost. DNA vaccines and vaccines based on conserved proteins alone performed poorly in pigs as opposed to mice. This shows that protecting mice against influenza is far more easy than protecting pigs and that truly universal influenza vaccines may well be wishful thinking. MLV vaccines that are stably attenuated by reverse genetics, such as NS1 mutants and elastase mutants, are more promising SIV vaccine candidates, but both their safety and efficacy need to be studied better. MLV

vaccines have the potential to induce a broader and more durable immunity than the killed vaccines, but there are no comparative studies of both types of vaccine and hard proof for the claim of broad protection is still lacking. It is not very realistic to expect that any MLV vaccine will induce a solid cross-protection between H1 and H3 SIVs, or an effective immune response in young pigs with MDA, if even infection with live wild-type SIV fails to do so. In addition, it is possible that preexisting active immunity will interfere with the replication of MLV vaccines and reduce their effect.

5 Conclusions and Outlook

We face two major questions. Can we define consistent criteria for replacement or addition of virus strains in the available inactivated SIV vaccines? Is it time to start using MLV vaccines for influenza in pigs? We need to think outside the box to find the best possible answer to both questions. The commercial inactivated SIV vaccines are adjuvanted and they are obviously more drift resistant than the nonadjuvanted human influenza vaccines. Some of the criteria for updating human or equine vaccines are therefore less useful for swine vaccines; e.g., genetic drift or poor reactivity of contemporary SIVs in HI tests with post-infection sera to previously circulating strains. HI antibody titers with post-vaccination sera of pigs vaccinated with existing vaccines may be better predictors of protection, but the results will also depend on the adjuvant formulation and the amount of antigen in the vaccine, factors that are not standardized in the commercial SIV vaccines. From the regulatory viewpoint, the rigorous licensing requirements for SIV vaccines in Europe remain a hurdle for vaccine strain updates, should they be needed. Alternative MLV approaches for SIV will continue to be developed (Pena et al. 2011), because of the increasing use of reverse genetics technology. An in-depth analysis of the safety of these vaccines is essential. MLV vaccines like the NS1 vaccines discussed in this chapter aim to stimulate an immune response more closely resembling that induced by natural infection, but their ability to do so or to represent an improvement over killed vaccines remains to be proven. There is no doubt that NS1 or other MLV vaccines will still need to be multivalent and it is questionable whether they can be effective in the presence of MDA. Another key question is how these vaccines will perform in pigs with multiple prior exposures to SIV. Such preexisting immunity to influenza virus clearly boosts and broadens the immune response to inactivated vaccines but may interfere with the replication of live influenza vaccine, as it does in humans. In humans, live vaccine appears to be more effective than killed vaccine as a priming vaccine, and killed vaccine appears to be more effective in boosting preexisting immunity (Ambrose et al. 2011; Dormitzer et al. 2011; Fiore et al. 2009). Also in pigs, the best vaccine approach may differ for young piglets and feeder pigs as opposed to influenza-primed breeding animals.

The greatest limitation is the multitude of unanswered questions about the pig's immune response to SIVs and different types of SIV vaccines. Which amino acids in the HA of SIVs are most important for antibody binding and drift? May the combination of multiple influenza virus strains in one vaccine result in "antigenic competition" and a reduced or biased antibody response? How do adjuvants affect the functional antibody response? What is the true protective value of immune responses other than neutralizing antibody to the HA? How durable and solid is the broad protection observed in experimental infection studies? May killed vaccines with heterologous strains have a greater chance to be efficacious in pigs with MDA to field strains? These are just a few examples. One positive side effect of the 2009 pH1N1 virus is that it has, at least temporarily, increased the interest in both SIV surveillance and research. Surveillance is essential to get a picture of the dominant SIV subtypes and lineages in different geographic regions, to detect changes, and to assist with vaccine strain selection. Several of the research questions mentioned here also hold true for humans. What is more, studies of the immune response to (human) influenza viruses in the pig may yield insights and information that are important for both veterinary and human medicine.

Acknowledgments The authors would like to acknowledge grant support from the National Institute of Allergy and Infectious Diseases, National Institute of Health, Department of Health and Human Services (Contract No. HHSN266200700005C), the European Commission (FP7—GA258084, FLUPIG), and the Belgian Federal Public Service for Health, Food Safety and Environment (RT 09/6227, FLUCROSS).

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Swine Influenza Virus Infections in Man

Whitney S. Krueger and Gregory C. Gray

Abstract Because pigs are susceptible to both avian and human influenza viruses, genetic reassortment between avian, human, and/or swine influenza viruses in the pig host can lead to the generation of novel influenza A viruses (Ma et al. 2009). Since the first serological evidence of a swine influenza virus (SIV) infecting humans in 1958, sporadic cases have continued to occur. In recent years, case reports have been increasing, seemingly in concert with modern pig farming and the emergence of triple reassortant SIVs in swine. SIV infections in man generally are mild or subclinical, and often are not diagnosed; however, SIV infections can be quite serious in patients with underlying medical conditions. As of August 2010, 73 case reports of symptomatic human SIV infections have been documented in the medical literature or reported by health officials (excluding cases of the 2009 pandemic H1N1 influenza virus), of which 7 infections (10 %) resulted in death. While exposure to swine is often considered a risk factor for human SIV infections, 37 of 73 (51 %) reported cases had no known exposure to pigs; consequently, SIV may be crossing the species barrier via transmission routes yet to be acknowledged. In addition, human-to-human transmission was suspected in 10 of 34 (30 %) of the cases with epidemiological investigation. This chapter discusses the observations of illness and infections in humans, risk factors associated with infection, and methods for diagnosing human infections of SIV.

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Current Topics in Microbiology and Immunology (2013) 370: 201–225
DOI: 10.1007/82_2012_268

Published Online: 29 September 2012

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1 Historical Background

Influenza-like illness in pigs was first recognized during the fall wave of the 1918 human pandemic, for which historical reports described serious illness and deaths among pigs at the Cedar Rapids Swine Show in Iowa, from September 30 to October 5, 1918 (Zimmer and Burke 2009; Laidlaw 1935; Shope 1936). Pigs that survived the show and returned to their home farms further spread the illness to pigs that did not attend the show. Soon thereafter, the disease was widespread in swine herds in the Midwest, and to a varying extent, the epizootic reappeared every winter season. What researchers suspected as "flu", due to the similarity of symptoms with human influenza, Robert Shope confirmed in the 1930s, when he transmitted infectious agent from sick pigs to healthy animals and demonstrated that human and swine pathogens were closely related, as human sera neutralized the swine virus (Shope 1931, 1936). This classical H1N1 SIV continues to circulate among pigs in Asia and America.

In 1958, the first serological evidence of SIV infecting humans was associated with a 40-year-old Czechoslovakian female laboratory worker with exposure to pigs (Kluska et al. 1961; Myers et al. 2007). An epidemiological investigation revealed probable human-to-human transmission, as five family members of the index case became ill with influenza symptoms and had serological evidence of exposure to the H1N1 SIV. The family members, ranging in ages from 3 to 72 years, reported no swine contact. Then in 1974 in Minnesota, SIV was first isolated from a human after a 16-year-old male pig farmer with Hodgkin's disease died of pneumonia (Smith et al. 1976). Classical H1N1 SIV was isolated from the young man's lung post mortem.

Since the first well-documented reports in 1958 and 1974, sporadic human infections caused by SIVs have continued to occur, with about one US case reported to the Centers for Disease Control and Prevention each year; however, sustained viral transmission among humans rarely follows, as viral adaptation to the human host requires significant (and currently unidentified) combinations of viral genes (Van Reeth 2007). While little is known about factors involved in avian and human influenza virus adaptation to swine, even less is known about SIV adaptation to humans. Evidence suggests that influenza A viruses' human or animal host discrimination is a polygenic trait (Kuntz-Simon and Madec 2009).

In Wisconsin in 1994, zoonotic transmission of SIV to two laboratory research personnel caring for pigs experimentally infected with A/Sw/IN/1726/88 provided new insight into SIV infections in man (Wentworth et al. 1997). The two laboratorians developed mild influenza symptoms after collecting nasal swabs from the infected pigs while following biosafety level 3 biocontainment practices. The influenza viruses isolated from the workers were direct descendents of the inoculum virus and antigenically identical. No differences were found with HA and NA gene sequence analyses of viruses isolated from each of the laboratorians; in addition, sequence analysis of the remaining influenza gene segments did not indicate viral reassortment had occurred. While this report concluded that zoonotic transmission of SIV did not require HA or NA gene mutations or viral reassortment, the potential still exists, and human infections with SIV should be monitored for viral reassortment and related mutations.

In previously healthy persons, human infections with SIV typically have caused mild respiratory disease and influenza symptoms. Complications with infection have resulted, most often when patients had preexisting medical conditions with weakened immune systems, although exceptions have been reported. SIV infections in humans have been detected in North America, Europe, and Asia (Table 1); however, it can be assumed that human infections with SIV are often unrecognized, as the illness can be asymptomatic or mild, and are very similar to infections with human influenza viruses. Human SIV infections documented in the medical literature or reported to the US CDC or international Ministries of Health between 1958 and 2009 had a case fatality rate of 10 % (7 of 73) and human-to-human transmission was suspected in 30 % (10 of 34) of the cases with epidemiological investigation. Sporadic cases continue to occur; however, since December 2005, case reports have been increasing, seemingly in concert with the emergence of triple reassortant SIVs in swine, the continual genetic evolution of these viruses, and larger swine confinement facilities, although this could also be attributed to better diagnostic capabilities and increased surveillance efforts (Fig. 1).

Table 1 Characteristics of reported symptomatic cases of humans infected with Swine Influenza viruses

Years	Country/State	Virus	Sex	Age	Swine Exposure	Suspected Cases by Serology	Outcome (immunocompetency)
1958	Czechoslovakia	H1N1 [a, b]	F	40 yr	Laboratory worker (index case)	5 family members	Influenza symptoms
			M	11 yr	None	Family member of index case	Influenza symptoms
			F	3 yr	None	Family member of index case	Influenza symptoms
			F	7 yr	None	Family member of index case	Influenza symptoms
			F	72 yr	None	Family member of index case	Influenza symptoms
			M	44 yr	None	Family member of index case	Influenza symptoms
1974	Minnesota	H1N1	M	16 yr	Swine farmer	No evidence	Fatal pneumonia (Hodgkin's disease)
1975	Wisconsin	H1N1[a]	M	8 yr	Lived on swine farm with SIV seropositive pigs	5/7 family members, all had pig contact	Influenza symptoms
	Virginia		F	40 yr	Fed pigs	No evidence	Pneumonia
	Virginia		M	55 yr	None	No evidence	Pneumonia (splenectomy, chronic bronchitis)
	Tennessee		M	17 yr	Meat-packing house worker	No evidence	Pneumonia (Hodgkin's disease)
1976	New Jersey	H1N1	M	18 yr	None; 13 military soldiers, 5 confirmed by isolation	230 military personnel	1 fatal case
	Missouri	H1N1[a]	M	32 yr	None	No evidence	Influenza symptoms
	Wisconsin	H1N1	M	23 yr	Swine farm worker exposed to SIV-positive pigs	No evidence	Influenza symptoms
	Wisconsin		M	13 yr	Lived on swine farm with SIV-positive pigs	Schoolmate	Influenza symptoms
	Minnesota		M	Unk	Swine exposure	N/A[e]	Unknown
1979	Texas	H1N1	M	20 yr	Livestock show swine barn worker	Roommate	Influenza symptoms
1980	Texas	H1N1	M	6 yr	Livestock show visitor; no direct contact with pigs	No evidence	Influenza symptoms
1982	Nevada	H1N1	F	5 yr	None	5/62 case contacts	Fatal pneumonia (Acute lymphoblastic leukemia in remission)

(continued)

Table 1 (continued)

Years	Country/State	Virus	Sex	Age	Swine Exposure	Suspected Cases by Serology	Outcome (immunocompetency)
1983	Russia	H1N1	M	65 yr	Occupational exposure	No evidence	Died
			F	10 yr	None	No evidence	Unknown
			M	27 yr	None	No evidence	Unknown
1986	Switzerland	H1N1	M	50 yr	Occupational exposure to SIV-positive pigs	N/A	Severe pneumonia (asthma)
	Switzerland		Unk	3 yr	None	N/A	Influenza symptoms
	Netherlands		M	29 yr	Occupational exposure to ill SIV seropositive pigs	Father, had pig contact	Severe pneumonia (immunocompetent)
1988	Wisconsin		F	32 yr	Exposed to ill pigs at county fair	Several hospital care takers	Fatal pneumonia (36 wks pregnant)
1991	Maryland		M	27 yr	Laboratory animal caretaker exposed to ill pigs	Household contact	Fatal pneumonia
1992	Netherlands	H3N2	F	1 yr	None	Father	Mild respiratory disease
1993	Netherlands	H3N2	M	2 yr	None (father worked on pig farm)	No evidence	Mild respiratory disease
	Netherlands	H1N1	F	5 yr	Lived on swine farm	N/A	Severe pneumonia
1994	Wisconsin	H1N1	M	39 yr	Laboratory worker; experimentally infected pigs	No evidence	Influenza symptoms
			F	31 yr	Laboratory worker; experimentally infected pigs	No evidence	Influenza symptoms
1995	Minnesota	H1N1	F	37 yr	Occupational exposure to ill pigs	N/A	Fatal pneumonia (Immunocompetent)
1998	United States	H1N1[c]	Unk	Unk	Unknown	N/A	Unknown
1999	Hong Kong	H3N2	F	10 mo	None	No evidence	Influenza symptoms
2001	Hong Kong	H1N1	F	4 mo	Unknown	N/A	Mild respiratory disease
2002	Switzerland	H1N1	M	50 yr	Swine farmer	No evidence	Influenza symptoms

(continued)

Table 1 (continued)

Years	Country/State	Virus	Sex	Age	Swine Exposure	Suspected Cases by Serology	Outcome (immunocompetency)
2004	Philippines	H1N2	M	25 yr	None	No evidence	Influenza symptoms
2005	Thailand	H1N1	M	4 yr	None	No evidence	Influenza symptoms
	Wisconsin	H1N1[c]	M	17 yr	Slaughterhouse	No evidence	Upper respiratory illness
	Iowa	H1N1[c]	M	50 yr	Swine farmer	No evidence	Influenza symptoms
	Canada	H3N2[c]	M	Unk	Swine farm worker exposed to ill pigs	N/A	Influenza symptoms
2006	Missouri	H1N1[c]	M	7 yr	None	N/A	Mild respiratory disease (immunodeficient)
	Iowa		F	4 yr	None	3 family members	Hospitalized for influenza symptoms
	Canada	H3N2[c]	M	7 mo	Lived on farm with SIV seropositve pig	4 household and 4 community members	Mild respiratory disease
2007	Ohio	H1N1[c]	F	10 yr	Exhibited pigs at swine fair; ill pigs at fair	N/A	Influenza symptoms
	Ohio		M	36 yr	Exhibited pigs at swine fair; ill pigs at fair	N/A	Influenza symptoms
	Illinois		F	48 yr	Visited fair, in vicinity of pig pen	N/A	Pneumonia, respiratory failure (asthmatic)
	Iowa		M	2 yr	Lived on swine farm	N/A	Influenza symptoms
	Michigan	H1N2[c]	M	16 mo	Visited fair, came within 1 m of pigs	N/A	Hospitalized for influenza symptoms
	Canada	H3N2[c]	M	6 yr	Lived on swine farm	Father and brother (likely pig contact)	Influenza symptoms and acute parotitis
2008	Spain	H1N1	F	50 yr	Swine farmer	No evidence	Influenza symptoms
	Minnesota	H1N1[c]	F	26 yr	Visited live-animal market	N/A	Pneumonia, sepsis, and respiratory failure
	Texas		M	14 yr	Visited swine farm, brought home and handled pig	N/A	Influenza symptoms (asthmatic)
	South Dakota		M	19 yr	None	8 case contacts	Influenza symptoms

(continued)

Table 1 (continued)

Years	Country/State	Virus	Sex	Age	Swine Exposure	Suspected Cases by Serology	Outcome (immunocompetency)
2009	Iowa	H1N1[c]	M	3 yr	Visited swine farm with ill pigs	N/A	Influenza symptoms (eczema)
	California	H1N1[d]	M	10 yr	None	N/A	Influenza symptoms
			F	9 yr	None	N/A	Influenza symptoms
	Canada	H1N1[c]	M	37 yr	Large hog operation worker	N/A	Influenza symptoms
			M	42 yr	Large hog operation worker	N/A	Influenza symptoms
			F	28 yr	Large hog operation worker	N/A	Influenza symptoms
	Kansas	H3N2	Unk	Unk	Contact with pigs at county fair; child	N/A	Influenza symptoms
	Iowa		M	Unk	None; child	No evidence	Influenza symptoms

[a] Diagnosis by serology, not the standard of virus isolation

[b] All H1N1 viruses isolated from humans in Europe were avian like; H1N1 viruses isolated from humans in the United States were "classical"

[c] Triple reassortant virus

[d] Quadruple reassortant virus. First identified cases of pandemic H1N1 (A/California/07/2009), thought to have originated in Mexico

[e] Epidemiological investigation not conducted or reported

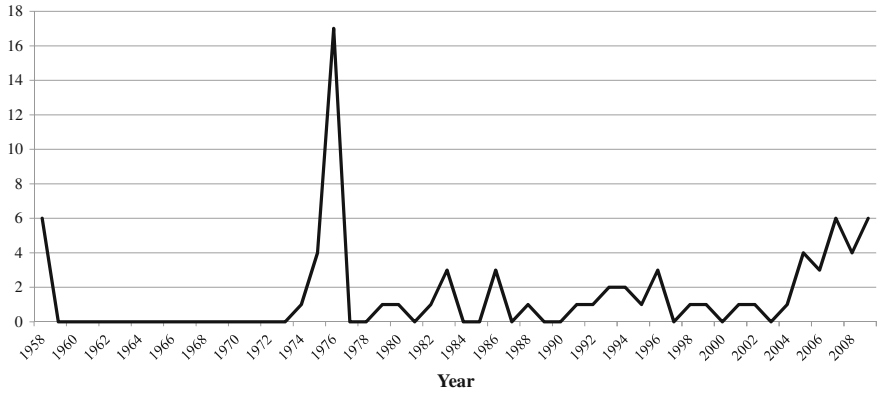

Fig. 1 Number of SIV infections diagnosed in humans, by year (1958–2009). (Not including human cases of the 2009 pandemic H1N1 Influenza virus)

2 SIV Infections in Man 1970s–1990s

2.1 North America

Subsequent to the isolation of SIV from a pig farmer in 1974, sporadic US case reports of illness caused by SIV continued. Up until 1998, all human infections in the United States with SIV were caused by the classical H1N1 virus. In 1975, four cases with ages ranging from 8 to 71-years-old were diagnosed with H1N1 SIV by serology in Wisconsin, Virginia, and Tennessee (O'Brien et al. 1977; Thompson et al. 1976; Smith 1976; McKinney et al. 1990). The Wisconsin case was an 8-year-old boy living on a swine farm, for whom a seroepidemiological follow-up investigation revealed H1N1 SIV seropositive pigs, as well as SIV infections in five out of seven family members who also had swine contact (all asymptomatic). Two adults in Virginia both developed pneumonia following SIV infections: an immunocompetent female who fed pigs and an immunosuppressed male (due to splenectomy) who had no known swine contact. No secondary cases were identified. A retrospective examination identified the fourth 1975 case in a 17-year-old male with Hodgkin's disease who worked in a meat-packing house and had contact with pigs. He developed pneumonia but survived. Serological results suggested that the male was infected with a New Jersey/1976-like SIV, the cause of a future human SIV outbreak in 1976.

The first large-scale occurrence of human-to-human transmission of SIV occurred at a military base in Fort Dix, New Jersey from January 19 through February 9, 1976, although exposure to pigs was never documented (Top and Russell 1977; Gaydos et al. 1977b). Thirteen male soldiers, with an average age of 18 years, were serologically diagnosed with classical H1N1 SIV infection; five cases were confirmed by viral isolation (Gaydos et al. 1977a, b, 2006; Hodder et al.

1977). One soldier experienced fatal pneumonia. A subsequent serological investigation at Fort Dix identified 230 additional suspected cases. Because of this outbreak, a nation-wide vaccine campaign was initiated in the US. After 10 weeks and 45 million people vaccinated, the campaign was halted due to the lack of evidence of virus transmission outside the military base and a suspected association between the vaccine and cases of Guillain–Barre syndrome (GBS) (Sencer and Millar 2006; Marks and Halpin 1980). However, little evidence exists to support an independent causal association between influenza vaccines and GBS, as most associations have only been temporal (Haber et al. 2009; Nachamkin et al. 2008; Evans et al. 2009). Nonetheless, as compared to other influenza A viruses, evidence for a causal association was strongest for this New Jersey/1796 swine influenza vaccine, with relative risks ranging from 4.0 to 8.0 and corroborated in multiple studies (Kurland et al. 1985; Safranek et al. 1991; Stratton 2004; Haber et al. 2009).

Four more cases unassociated with Fort Dix were reported between October and December 1976 in Missouri, Wisconsin, and Minnesota (Dowdle and Hattwick 1977). A New Jersey/1976-like infection was serologically diagnosed in Missouri in a 32-year-old male telephone lineman who reported no contact with pigs. An epidemiological investigation revealed no secondary transmission. In two separate incidents in Wisconsin, New Jersey/1976-like SIV was isolated from a swine farmer and an adolescent boy who lived on a swine farm. At the time of their illnesses, SIV was also isolated from pigs on each of the farms. In addition, a seroepidemiological investigation revealed a schoolmate of the boy had serological evidence of exposure to the same virus. In December, SIV was isolated from a man in Minnesota who reported contact with pigs.

In 1979 and 1980, New Jersey/1976-like SIV was isolated from two people in Texas, and both were associated with swine shows (Dacso et al. 1984). A 5-year-old girl with acute lymphoblastic leukemia (ALL) in remission died of fatal pneumonia as a result of a New Jersey/1976-like SIV infection in Nevada in 1982 (Patriarca et al. 1984). She reportedly had no pig contact, but an epidemiological investigation indicated five secondary cases resulted (out of 62 examined). The next documented SIV case was reported 6 years later in 1988 in Wisconsin and resulted in the death of a 32-year-old pregnant woman who came in contact with pigs at a county fair (McKinney et al. 1990; Wells et al. 1991). Then in 1991 in Maryland, a laboratory animal caretaker exposed to SIV-infected pigs died of pneumonia. Upon investigation, four other laboratory workers and one household contact of the case had serological evidence of SIV infection (Wentworth et al. 1994). Again in 1994, in Wisconsin another incident of laboratory exposure to experimentally infected pigs resulted in two human infections with influenza symptoms (Wentworth et al. 1997). In 1995, an occupational exposure to ill pigs led to an SIV infection and subsequent fatal pneumonia in a 37-year-old immunocompetent female in Minnesota (Kimura et al. 1998).

2.2 Europe

Since the first diagnosis of SIV in humans in Czechoslovakia, Europe has had 11 documented human cases of SIV infection. Unlike the United States, all H1N1 SIVs isolated from humans in Europe have been avian like. The first isolation of an SIV from a European occurred in Russia in 1983, after three people were diagnosed with H1N1 SIV infections (Chuvakova et al. 1985; Myers et al. 2006). A 65-year-old man had occupational exposure to pigs and died as a result of his infection. The other two cases, ages 10 and 27 years old, had no known swine exposure and survived. Three additional human cases of H1N1 SIV were diagnosed in early 1986 in Switzerland and the Netherlands. Both Swiss cases occurred in January, but in different regions of the country. In Central Switzerland, a 50-year-old asthmatic male with occupational exposure to pigs developed severe pneumonia but survived. In Western Switzerland, a 3-year-old child with no known exposure to pigs developed a mild respiratory disease. A follow-up seroepidemiological investigation indicated that an epizootic of SIV in pigs was concurrently occurring in the region where the child resided (de Jong et al. 1988). Then in March in the Netherlands, a Dutch immunocompetent male developed severe pneumonia following occupational exposure to ill pigs (de Jong et al. 1988). Two months following his illness, the pigs showed serological evidence of previous SIV infection; in addition, the man's father had serological evidence of infection.

Three additional cases in young children were diagnosed in the Netherlands in 1992 and 1993, but this time, two children living in two different regions were infected with a human–avian H3N2 reassortant SIVs, had no known exposure to pigs, and experienced mild respiratory disease (Claas et al. 1994; Rimmelzwaan et al. 2001). The father of one child regularly worked on a pig farm, but did not have an antibody titer against the virus. The other child's father had serological evidence of exposure to the virus isolated from his daughter. As of August 2010, this was the first and last occurrence of human infection with H3N2 SIV in Europe. The third child, who lived on a swine farm, was diagnosed with an H1N1 SIV infection and subsequently developed severe pneumonia but survived. The most recent human infections of SIV in Europe occurred in 2002 and 2008, in Switzerland and Spain, respectively, when H1N1 SIV was isolated from two 50-year-old swine farmers (Van Reeth and Nicoll 2009; Gregory et al. 2003; Adiego Sancho et al. 2009; ProMED-mail 2009a, b; 20 Feb).

2.3 Asia

Human infections with SIV have also been reported in Asia, albeit rarely. Three different SIV subtypes were responsible for four cases. First in 1999 in Hong Kong, H3N2 SIV was isolated from a 10-month-old girl who had no known swine exposure (Gregory et al. 2001). The virus was most closely related to European

strains of SIV. Then again in Hong Kong in 2001, this time an H1N1 SIV was isolated from a 4-month old girl for whom swine exposure was unknown (Chuang 2009). In the Philippines in 2004, H1N2 SIV was isolated from a 25-year-old male with no direct pig contact, and then in 2005, an H1N1 SIV was isolated from a 4-year-old boy in Thailand who had no direct pig contact. For both viruses, the hemagglutinin genes were similar to the classical Asia and North America swine H1 viruses, while the neuraminidase genes were more closely related to the European SIVs (Komadina et al. 2007).

3 Triple Reassorted SIVs Emerge in North America (1998–present)

Until 1998, classical H1N1 SIV was the only strain circulating in US pigs, and the only source for human infections in the US. In the late 1990s, in concert with the rapid growth of the US swine industry, a dramatic revolution in SIV reassortment was recognized. While swine and avian influenza virus reassortment, as well as swine and human influenza virus reassortment, had previously been documented, for the first time triple viral reassortment occurred in pigs. A reassortant virus emerged with H3N2 human influenza A surface glycoproteins and internal genes of swine, avian, and human influenza A viruses. The triple reassortant virus was isolated in US pigs from multiple outbreaks of respiratory disease (Zhou et al. 1999) and is now endemic in US swine and turkey populations (Yassine et al. 2007). Not long after the discovery of the H3N2 SIV, a human case of triple reassortant H1N1 SIV was recognized during molecular characterization of six SIV isolates collected from people between 1990 and 1998 (Cooper et al. 1999). A 1998 isolate, dubbed A/Wisconsin/10/98, contained a novel combination of classical swine, human, and avian influenza A genes.

Active human infections with triple reassortant SIV were first reported to the CDC in 2005 (Shinde et al. 2009). The first diagnosed case was an H1N1 triple reassortant SIV isolated from a 17-year-old male who was exposed to freshly killed pigs at a slaughterhouse in Wisconsin (Newman et al. 2008). An active, symptomatic H1N1 triple reassortant SIV infection was identified in a 50-year-old male swine farmer in Iowa who was participating in a prospective cohort study of influenza infections (Gray et al. 2007a). Also in 2005, the first H3N2 triple reassortant SIV was isolated from a male swine farm worker in Canada, as well as from sick pigs on his farm. This same virus was also linked to an epizootic in pigs and turkeys occurring throughout Canada at that time (Olsen et al. 2006). All of the 2005 cases experienced typical influenza-like illnesses and fully recovered.

In 2006, two human cases of triple reassortant H1N1 SIV were reported in Missouri and Iowa (ProMED-mail 2007; 8 Jan; Shinde et al. 2009). Both cases had no known exposure to pigs, even more, human-to-human transmission of the virus probably occurred with the Iowa case, as three family members were also suspected cases and the grandfather lived on a swine farm. Another occurrence

of human-to-human transmission was suspected in a 2006 incident in Canada (Robinson et al. 2007). Following a mild respiratory illness, an H3N2 triple reassortant SIV was isolated from a 7-month-old boy living on a communal farm that raised several species of animals, including pigs. A serological investigation of 53 of the 90 farm residents and 10 farm pigs indicated four of seven household members, 4 of 46 nonhousehold members, and 1 of 10 farm pigs were seropositive for SIV exposure. The boy had no direct contact with the farm animals; however, his father occasionally spent time in the swine barn, and his uncle, who lived next door, worked in the swine barn.

In 2007, four human cases of triple reassortant H1N1 SIV infections were reported: a father and daughter who exhibited pigs at an agricultural fair in Ohio, a 48-year-old female who visited a fair in Illinois, and a 2-year-old boy who lived on a swine farm in Iowa (Vincent et al. 2009; Shinde et al. 2009). Also in 2007, an H1N2 triple reassortant SIV was isolated from a 16-month-old baby after he visited a fair in Michigan and came within 1 meter of pigs (Shinde et al. 2009). A Canadian boy who lived on a swine farm was infected by an H3N2 triple reassortant (Bastien et al. 2009). His case was unique, in that he experienced acute parotitis, which is not typically a clinical sign associated with influenza illness. A follow-up serosurvey found that three of six household contacts had serological evidence of SIV infection.

Three more human cases of H1N1 triple reassortant SIV were reported in 2008 in Minnesota, Texas, and South Dakota (Shinde et al. 2009; ProMED-mail 2008; 25 Nov; SDDoH 2009). The Minnesota case had visited a live-animal market with pigs and the Texas case had visited a swine farm and even brought a pig home with him. The South Dakota case was a 19-year-old college student with no known exposure to pigs. Following his illness, an epidemiological investigation indicated eight additional suspected cases by serology.

In April 2009, a novel quadruple reassortant H1N1 SIV was detected in two children in California. The virus contained genetic material from classical swine H1N1 (HA, NP, NS genes), North America H3N2 triple reassortant H3N2 (PB2, PB1, PA genes), and avian-like Eurasian swine H1N1 (NA, M genes). This novel H1N1 influenza virus ultimately spread worldwide causing a mild influenza pandemic in 2009–2010.

Separate from the cases of the novel H1N1 pandemic SIV strain, reports of human SIV infections *unrelated* to the pandemic strain still surfaced in 2009. In February before the pandemic, a 3-year-old Iowan boy, who had visited a family swine farm and had close contact with ill pigs, developed an H1N1 triple reassortant SIV infection (CDC 2009). He experienced mild influenza symptoms and no additional cases were identified. Then in Canada in June, three hog operation workers were diagnosed with an H1N1 triple reassortant SIV that contained hemagglutinin and neuraminidase genes similar to that of seasonal influenza virus (A/Bribane/59/2007) (Bastien et al. 2010). Researchers theorized that a triple reassortant SIV that had been circulating in pigs since the late 1990s, and the seasonal human H1N1 influenza virus had undergone genetic reassortment to create this progeny virus. Also, two human cases of an H3N2 SIV were reported: a child in Kansas who had contact with pigs at a county fair (ProMED-mail 2009; 8 Aug), and a child in Iowa with no known contact with pigs (ProMED-mail 2010; 16 Jan).

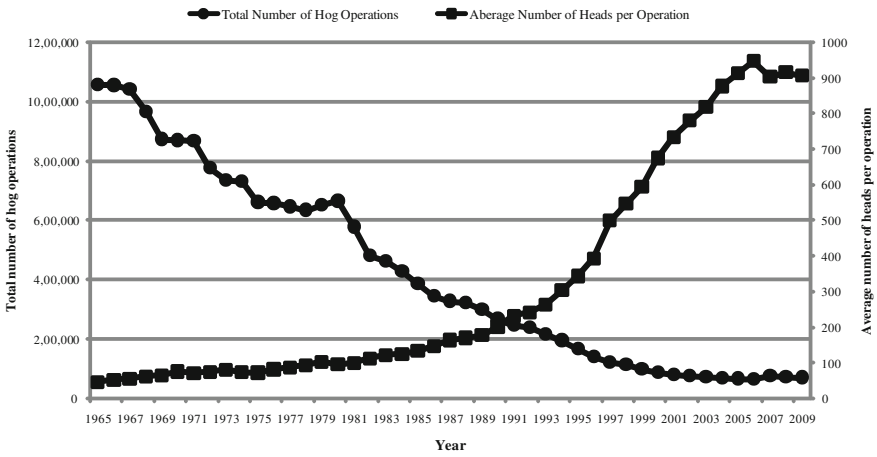

Fig. 2 Trends in hog operations in the United States (1965–2009). *Source: United States Department of Agriculture's National Agricultural Statistics Service 2010*

4 Epidemiology and Risk Factors

4.1 Modernized Farming Practices and Confined Animal Feeding Operations

Swine production practices in the United States have changed significantly in the last 50 years. Modern industrialized production facilities have emerged and replaced the traditional independent small-scale farm (Fig. 2) (NASS 2010). In the past, pigs were kept as an adjunct to cropping ventures and used to add value to corn when prices dropped. When the swine enterprise began offering significant returns, the modern swine industry became specialized, farm sizes grew, and productivity was optimized (Pitcher 1997; MacDonald 2003).

In 2008, operations with more than 5,000 head accounted for 88 % of the pig crop (NASS 2009). Such large numbers of susceptible pigs housed closely together in confinement affords a perfect opportunity to spread infectious diseases; consequently, and predictably, despite biosecurity measures, SIV is now endemic among these modern production facilities (Van Reeth et al. 2008; Poljak et al. 2008). Even more, this optimization in productivity daily exposes immunologically na workers to thousands of pigs potentially harboring SIVs, which presents an opportunistic setting for humans to serve as an interface or bridging population for the cross-species sharing of influenza viruses and generation of novel viruses due to the risk of viral reassortment. The 2009 event in Canada illustrates this potential, as the virus that infected three hog operation workers was a result of genetic reassortment between an SIV endemic in swine herds and the seasonal human H1N1 influenza virus (Bastien et al. 2010). Even with the growth of the swine industry, small backyard farming continues to be a viable enterprise in some rural communities. Although backyard pig

exposures are less intense and shorter than those in a confined animal feeding operation (CAFO) setting, the risk of virus sharing between humans and pigs persists, especially when biosecurity is lax (Gray and Kayali 2009).

4.1.1 Animal Worker's Occupational Risk of SIV Infection

Several cross-sectional seroprevalence studies have examined swine workers' increased risk of exposure to SIVs. A 2002 study reported that modern swine workers were significantly more likely to have elevated antibodies against novel SIVs, in comparison to controls not exposed to swine (Olsen et al. 2002). A 2006 study found that swine farmers, swine veterinarians, and pork processing workers were significantly more likely to have elevated antibodies against classical H1N1 and emergent H1N2 SIVs. The study also reported the adjusted odds ratio (OR) for swine farmers having an elevated antibody titer to a classic H1N1 SIV was 35.3 (95 % CI 7.7-161.8) in comparison to controls not exposed to swine (Myers et al. 2006). In another 2006 publication, swine workers' high risk (OR = 30.3; 95 % CI 3.8-243.5) of elevated antibodies to H1N1 SIV diminished to almost that of nonexposed controls when the workers reported using gloves during their occupational exposures (Ramirez et al. 2006). Swine workers who self-reported smoking were at increased risk of SIV infection, suggesting the possibility of oral autoinoculation when workers smoked cigarettes. Another study estimated that antibodies to SIV may be present in up to 23 % of humans with occupational exposure to pigs (Van Reeth 2007).

A 2007 2 year prospective study of 803 rural Midwesterners validated these cross-sectional study reports (Gray et al. 2007a). While swine farmers had markedly elevated odds for increased antibody titers against swine influenza viruses, indicating previous occupational infection, prospective data revealed serological and culture evidence of SIV infection over the 2 years of follow-up, as one of the first identified H1N1 triple reassortant SIV was isolated from a study participant with influenza symptoms. Furthermore, many of the swine workers' spouses, 90 % of whom denied having direct contact with pigs, also had increased antibody titers against SIVs. This capacity for secondary or fomite transmission illustrates the precarious potential for swine workers to function as a bridging population between man and pigs and introduce SIV to their communities. As half of the reported human cases of SIV infection reported no direct contact or exposure to pigs, secondary transmission by person or fomite is almost certainly the cause for the majority of these infections.

Swine industry workers in the US often are immigrants with English as their second language, often do not have ready access to medical care in their rural communities, and may shun contact with public health authorities due to questions regarding their immigration status. Due to these barriers to medical care, the detection of novel viruses may be missed or greatly delayed. Therefore, to reduce the likelihood of cross-species influenza virus transmission, swine workers need to be considered as having high risk of SIV infections and protected as much as possible (Gray and Baker 2007; Gray and Kayali 2009; Gray et al. 2007b). Employers should train workers in wearing personal protective gear like gloves, not smoking when working with pigs, and require workers to seek medical screening upon development of influenza-like illnesses. National

pandemic plans should acknowledge swine workers as a priority group for receiving annual seasonal influenza vaccines, pandemic influenza vaccines, antivirals, and also include such workers in influenza surveillance efforts.

4.1.2 Intensive Swine Production and SIV

SIV is endemic where modern production facilities are common (Van Reeth et al. 2008). The United States Department of Agriculture's (USDA) National Animal Health Monitoring System (NAHMS) conducted a seroprevalence study in 2000 of a stratified random sample of production pigs in 17 states (representing 94 % of the US pork industry) (Bush et al. 2003). Based on SIV seroprevalence in the study's sample of finisher herds, it was estimated that 49.8 % of the nation's finisher herds were SIV-infected herds. In addition, 41.9 % of herds were susceptible to SIV infection as vaccines were not administered and there was no serological evidence of previous infection. Only 8.3 % of herds were protected from SIV infection due to vaccination. In 2001, H1N1 SIV seroprevalence among 65 sow herds and 72 finisher herds in Canada was 61.1 % and 24.3 %, respectively (Poljak et al. 2008). In 2006, USDA's NAHMS repeated their serological survey of SIV among US swine herds in 17 states (APHIS 2009). Among unvaccinated herds, 71.5 % of the herds were seropositive for either H1 or H3 SIV. Risk factors for sow-herd SIV seropositivity included pig density, an external source of breeding pigs, total animals on site, and closeness of barns. Risk factors for finisher-herd SIV positivity included SIV-positive sows, a large herd size, high pig farm density, and farrow-to-finish farm type (Poljak et al. 2008).

Several industry limitations have prevented timely and accurate surveillance for emerging SIVs. SIV surveillance in pigs is largely passive and voluntary; active surveillance only involves sentinel events such as human illness with pig exposure or very sick pigs. However, pigs do not always have clinical signs of novel virus infection. In addition, neither industry nor government has established a compensation system to protect swine farmers in the event that they have a herd infected with a novel SIV. Because of these limitations, swine workers who will likely have antibodies against enzootic SIVs should be monitored, such that an influenza-like illness would indicate infection with a new virus.

As SIVs are endemic in swine herds across the world, viral reassortment between circulating strains has occurred in the past and will continue to occur. For example, in 2006 a reassortant H2N3 SIV was isolated from US pigs that had HA, NA, and PA gene segments of an American avian influenza virus and other gene segments of an emerging triple reassortant SIV that contained human, avian, and swine influenza virus gene segments (Ma et al. 2007). This was a significant discovery because the H2N2 influenza subtype had recently only been seen in birds and had not circulated in humans since 1968; therefore, people born after 1968 had no immunity to this subtype. Continued circulation of the H2N3 SIV among pigs could afford an opportunity for this avian-swine reassortant virus to

better adapt to mammalian species. Fortunately, a serologic survey of swine workers exposed to pigs infected with the emergent H2N3 SIV indicated humans were not infected with this virus (Beaudoin et al. 2010).

During the summer of 2009, the pandemic H1N1 virus was first discovered in swine herds in Canada and show pigs at an agricultural fair in Minnesota (Pasma and Joseph 2010; Gray et al. 2012). Just 1 year since its discovery in pigs, this virus was endemic in swine herds (Vijaykrishna et al. 2010). In August 2010, the World Organization for Animal Health (OIE) estimated the pandemic virus was circulating in pigs in numerous countries spanning North America, South America, Europe, Asia, and Australia. And as anticipated, viral reassortment soon followed (Vijaykrishna et al. 2010). In January 2010, a novel SIV appeared in pigs in Hong Kong with the pandemic H1N1 neuraminidase gene, European avian-like H1N1 hemagglutinin gene, and triple reassortant H1N2 internal genes. The latter two lineages had been circulating in Hong Kong pigs for the past 10 years. Consequently, there is a real risk that this pandemic strain will further reassort with currently circulating SIVs to create a novel, more virulent progeny virus capable of again infecting humans.

4.2 Swine Shows at Agricultural Fairs

In recent years, swine shows at state and county agricultural fairs have been recognized as playing an important role in viral transmission between pigs and humans, as 8 (15 %) of the 52 total SIV infections diagnosed in the United States have been linked to pig exposures at agricultural fairs. Yet even historically, in 1979 and 1980, two HINI SIV (New Jersey/1976-like) cases were diagnosed in Texas and associated with fairs (Dacso et al. 1984). A 20-year-old male college student developed influenza symptoms while working at a livestock show swine barn, and reportedly sleeping in the livestock area with 2000 pigs. Secondary transmission to his roommate was suspected, as indicated by serology results. The second case, a 6-year-old boy, had attended a large regional livestock show and visited the swine barn, although he reportedly never touched a pig. No additional cases or secondary transmission were identified.

In September 1988, after visiting a county fair swine exhibition, a previously healthy 32-year-old pregnant woman (36 weeks) died of pneumonia following complications of an H1N1 SIV infection (Wells et al. 1991). Show pigs had influenza-like illnesses (ILIs) during the fair, and it was reported that several of the swine exhibitors also experienced ILIs in September 1988. A follow-up serological investigation found 19 (76 %) of 25 swine exhibitors had elevated serum SIV antibody titers, as well as the woman's husband who also attended the fair. Even an exhibitor's two young siblings who attended the fair developed ILIs within 5 days of exposure and had elevated SIV titers. One was diagnosed with pneumonia. Further investigations suggested human-to-human transmission, as several health care personnel caring for the patient developed ILIs within 5 days after exposure to the patient and had laboratory evidence of SIV infection by serology.

There was an association between reporting ILI and having an elevated SIV antibody titer (RR, 7.0; 95 % CI, 1.2 to 53).

In 2007, four human cases of triple reassortant SIV (3 H1N1 and 1 H1N2) were linked to swine fairs. In Ohio, SIV was isolated from a father and 10-year-old daughter with influenza symptoms, who had exhibited pigs at a swine fair. Reportedly two-thirds of the 235 market pigs in the swine barn were clinically ill, and 26 people who had close contact with the show pigs developed ILIs (Vincent et al. 2009). In Illinois, a woman visited a fair but was only in the vicinity of the pigpens and did not have direct pig contact (Shinde et al. 2009). In Michigan, a 16-month-old baby boy was diagnosed with a triple reassortant H1N2 SIV infection after visiting a swine fair and coming within 1 meter of the pigs. More recently, a 2009 case of an H3N2 SIV infection in a child has also been linked to pigs at a county fair in Kansas (ProMED-mail 2009; 8 Aug). All cases experienced influenza symptoms and recovered uneventfully.

Asymptomatic, and thus unapparent, SIV infections in the show pigs, along with the tight housing quarters and close contact participants have with the pigs provide an opportunistic setting for the cross-species sharing of influenza viruses. Even more, while fair regulations do prohibit the presence of symptomatic pigs with respiratory illness (either by quarantine or expulsion), the time of viral shedding and clinical symptoms do not coincide. Once a pig shows signs of illness, influenza virus has already been shed into the barn environment. In August of 2009, after the pandemic H1N1 was circulating among human populations in the United States, the virus was first identified in seemingly healthy US pigs at a swine show in Minnesota (Gray et al. 2012). Evidence suggests that the pigs came to the show already infected and shedding, thus indicating that the pandemic virus was already established in the US swine population prior to the show. Even more, there is epidemiological evidence that a father and daughter showing one of the infected pigs developed influenza-like illnesses while attending the fair, indicating swine-to-human transmission of the virus, although samples were not collected.

4.3 Laboratory Exposures to SIV

Laboratory exposures to experimentally infected swine have resulted in four documented human infections with SIV. In addition to the very first serological evidence of SIV infection in humans occurring in a Czechoslovakian laboratory worker in 1958, three additional cases of H1N1 SIV infection occurred in the United States in the early 1990s. Two laboratory workers were infected with an H1N1 SIV after collecting nasal swabs from experimentally infected pigs, even while using biosafety level 3 biocontainment practices. A previously healthy laboratory animal caretaker died of pneumonia after exposure to sick pigs (Wentworth et al. 1994). The influenza virus was antigenically related to, but still distinguishable from, SIVs circulating at that time; however, viral isolates were not collected from the sick or dead pigs with

which the case had direct contact. These reports illustrate the risk of viral transmission while working with SIV in a laboratory setting.

5 Diagnosis of SIV in Humans

Along with demonstrating influenza symptoms, including fever and cough or sore throat, recent exposure to pigs is often required for the differential diagnosis of a probable human SIV infection. As discussed above (and shown in Table 1), half of the reported human cases of SIV infections had no known exposure to pigs; hence, it can be assumed that many SIV infections are not diagnosed. Most developing countries do not have the resources available to conduct comprehensive diagnostic testing; moreover, many US clinical or county public health diagnostic laboratories only have the capability to employ simple rapid tests for human influenza A and B viruses. While some rapid influenza diagnostic tests are able to distinguish between influenza A and B virus infections, often rapid tests cannot differentiate between influenza A subtypes. Seasonal influenza A viruses and the novel H1N1 pandemic strain can produce positive rapid test results; however, rapid tests do not readily detect classical H1N1 SIV. A confirmatory diagnosis is difficult and relies on more advanced tests performed by reference laboratories, including real-time reverse transcription polymerase chain reaction (rRT-PCR), viral culture, or serology. Diagnostic rRT-PCR assays test for generic H1, H3, and H5 influenza A viruses. When a respiratory sample is returned as a "non-typable" influenza virus, as can be the case for novel reassortant SIVs, it is then often sent to a reference laboratory like the US Centers for Disease Control (CDC) reference laboratory in Atlanta, Georgia for more comprehensive study.

5.1 Serology

Conventionally, serological confirmation of an SIV infection is often performed following the CDC's hemagglutination inhibition (HI) assay protocol (WHO/CDS/CSR/NCS 2002). A positive result is generally recognized as a four-fold rise in virus-specific neutralizing antibodies between acute and convalescent sera samples. Titer results are reported as the reciprocal of the highest dilution of serum that inhibited virus-induced hemagglutination of a solution of erythrocytes. For diagnosing patients in the clinical setting, serology is not ideal, as the lag time in collecting the convalescent blood sample (2–3 months) is too long and not practical. It is, however, a worthy tool for studies at the population level (Gray et al. 2007a; Myers et al. 2006; Olsen et al. 2002; Ramirez et al. 2006). Although, for seroepidemiological studies of infections that could have occurred years ago, it has been demonstrated that the acute SIV infection titer cut-points limit statistical power. Instead, a proportional odds model that examines a full range of antibody

titers can maximize power to detect risk factors (Capuano et al. 2007). The HI assay does have some limitations when employed for population-based studies (Gray et al. 2007a). Influenza antibodies can cross-react with other influenza strains; therefore, antibodies against human influenza viruses (naturally acquired or by vaccination) could react to SIVs used in an HI assay, and often statistical adjustments are necessary to control for such cross-reactivity. In addition, the influenza strain used in an HI assay must closely match the circulating SIV strains potentially infecting the subjects. Genetic differences between antibodies and the strain used in the HI assay can produce a negative result and inaccurate estimate of risk. For these reasons, there is a critical need for new serological assays to be developed that can both *rapidly* identify infections as well as *differentiate* between different influenza strains.

5.2 Molecular Studies

Currently, molecular studies of human respiratory samples using rRT-PCR assays are superior to serology when diagnosing active influenza infections. For rRT-PCR approaches, extracted RNA from respiratory samples (nasal, throat, nasopharyngeal, or gargles) are first screened for generic influenza A virus, then subsequently analyzed through individual rRT-PCR reactions to rapidly determine the hemagglutinin (HA) subtype. The CDC's rRT-PCR protocol is often the method followed. Conversely, another rRT-PCR strategy detects a conserved region of the influenza matrix gene to screen for all influenza A viruses and can detect 25 genetically different viral isolates obtained from humans, swine, birds, and horses (Fouchier et al. 2000). Further genotypic analyses can determine if the influenza virus is of swine origin (Karasin et al. 2000a, b, c, 2002, 2004; Olsen et al. 2003; Zou 1997).

If SIV is detected in a respiratory specimen by rRT-PCR, then an effort is made to isolate the virus in Madin-Darby Canine Kidney (MDCK) cell cultures for further molecular characterization and to better understand the virus's genetic lineage. One developmental prototype that demonstrates the utility of a DNA array-based virus identification tool is the "Mchip", a low-density microarray that, for a relatively low cost and a short time frame, can differentiate between seasonal human H1N1, classical swine H1N1, and 2009 pandemic H1N1 influenza virus strains (Dawson et al. 2006; Mehlmann et al. 2007; Moore et al. 2007; Heil et al. 2010).

6 Conclusions

The recent emergence of the 2009 pandemic influenza virus with considerable swine genetic heritage, along with the increasing number of SIV infections in man, suggest that novel SIVs will continue to cause sporadic infections in humans. The

high prevalence and variety of enzootic SIVs in modern CAFOs have potential to generate viruses capable of greater morbidity and more efficient transmission. No single intervention can prevent influenza viruses from reassorting, efforts can be made to better monitor and prepare for their emergence. Improved surveillance of swine and human populations for novel influenza viruses, along with wider seasonal vaccination coverage among agricultural workers, health care workers, and the general public are critical strategies in the battle against emerging zoonotic swine influenza viruses (Gray and Baker 2007; Gray and Kayali 2009; Gray et al. 2007b).

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Interspecies Transmission of Influenza A Viruses Between Swine and Poultry

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Abstract The special susceptibility of pigs to infection with avian and mammalian influenza viruses, the close proximity of pigs and poultry farms, and applied human practices in raising and trading of farm animals/farm animal products, provide opportunities for genetic exchange and interspecies transmission of influenza A viruses. Although only H1 and H3 influenza subtypes have widely circulated and caused disease in pig populations worldwide, H9 subtype is being continuously detected in pigs in Asia, plus sporadic infections with highly pathogenic H5-avian influenza viruses. On the other hand, swine viruses are continuously isolated from poultry species, especially turkeys, causing economic losses in poultry production. The viral and host factors contributing to influenza transmission between pigs and poultry are poorly defined. In addition, surveillance programs for influenza viruses in both species, especially pigs, are rarely implemented, and thus, leaving many questions about influenza unanswered. In this review, we summarize early and recent findings about influenza transmission between swine and poultry with emphasis on the role of turkeys.

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Current Topics in Microbiology and Immunology (2013) 370: 227–240
DOI: 10.1007/82_2011_180

Published Online: 14 December 2011

Contents

1 Brief About Influenza History in Pigs

Influenza was initially recognized in pigs in 1918, in a temporal and spatial coincidence with Spanish influenza in humans (Subbaroa et al. 2006). Diseased pigs manifested similar symptoms to those observed in humans suggesting a common etiology in both species. Retrospective serological testing revealed that the disease was caused by a closely related influenza virus in both species (Brown 2008). It was not until 1930 when the virus was isolated for the first time from pigs in North America, coinciding with the isolation of the virus from humans (Shope 1931a, b). The virus was then subtyped as H1N1 and recognized as classical H1N1 (cH1N1) lineage of swine influenza viruses.

cH1N1 swine viruses were almost exclusively the dominant cause of influenza in pigs in North America for nearly 70 years, remaining genetically and antigenically stable until 1998 (Vincent et al. 2008). Starting from 1998, pig populations were affected with a novel influenza that had not been recognized in North America before. The viruses were characterized of being double or triple reassortant H3N2 subtype with genes from human (HA, NA, and PB1), swine (NP, M, and NS), and sometimes avian (PB2 and PA) lineage viruses (Zhou et al. 1999). Consequently, H3N2 viruses reassorted with cH1N1 as well as human H1N1 viruses, resulting in a wide range of reassortant subtypes (reassortant H1N1 (rH1N1), H1N2, H3N1, and H3N2) that spread widely in pig populations in North America (Vincent et al. 2008).

Swine influenza viruses in Europe differ significantly from their counterparts in North America, both genetically and antigenically. Human H3N2 viruses were first detected in pig populations in Europe in the mid-1970s (Castrucci et al. 1994). Thereafter, avian H1N1 viruses transmitted to pig populations in the late 1970s, establishing stable lineages in the European continent (Scholtissek et al. 1983). Subsequently, avian-origin H1N1 reassorted with human-origin H3N2 resulting in new strains with surface glycoproteins being of human lineage viruses and internal genes from avian lineage viruses (Castrucci et al. 1993).

Although only viruses of H1 and H3 subtype have widely circulated in pigs, other influenza subtypes have been isolated from pigs worldwide, being mostly of avian origin. For example, avian H4N6 virus was isolated from a pig farm in Canada in 1999 (Karasin et al. 2000a, b). Additionally, avian H2N3 viruses were

isolated from two pig farms in the United States (U.S.) (Ma et al. 2007; Beaudoin et al. 2010). Serological and epidemiological evidences indicated the transmission of avian H4, H5, and H9 viruses to pigs in Asia (Peiris et al. 2001; Ninomiya et al. 2002).

2 Brief History of Influenza in Poultry

While most influenza infections in pigs result in high morbidity and low mortality, poultry species can be infected with highly pathogenic avian influenza (HPAI) resulting in high mortality (>70%) in the infected flock. Chickens and turkeys are not considered natural reservoirs of influenza viruses, however, avian and swine influenza viruses of various subtypes have been frequently isolated from both species worldwide (Alexander 2007; Swayne and Halverson 2007).

The highly lethal form of influenza disease was first recognized in chickens in Italy in 1887, and the causative agent was not identified as influenza virus until 1955 (Swayne and Halverson 2007). Until the middle of last century, H7 subtype was the dominant cause of highly pathogenic influenza in poultry species worldwide. In the late 1950s and early 1960s, viruses of H5 subtypes emerged as the second highly pathogenic form of avian influenza in poultry (Swayne and Halverson 2007). Currently, H5 and H7 are the only two out of 16 HA-identified subtypes that are considered HPAI, with H5 being the most spread form since 1998 causing tremendous losses in poultry production around the globe (Neumann et al. 2010; Stegeman et al. 2004).

Additionally, milder diseases can be associated with influenza infection in poultry species. It can be caused by any of the 16-HA subtypes and the viruses are designated as low pathogenic avian influenza (LPAI) (Swayne and Halverson 2007). Infection with such viruses results in little or no disease, with high morbidity and low mortality in some occasions. LPAI was first recognized in the middle of last century and the earliest isolate was recovered from chickens in Germany in 1949 and was not identified as influenza virus until 1960 (A/Chicken/Germany/49 (H10N7)) (Swayne and Halverson 2007).

Several influenza A subtypes have been isolated from chickens, turkeys, and other poultry species worldwide. Nonetheless, few subtypes have caused or continue to cause significant threat to poultry production, depending on the poultry species and its geographical location.

In 1997, H5N1 HPAI transmitted to poultry from wild birds and spread to many Asian, African, and some European countries (Cauthen et al. 2000; Aly et al. 2008). In South East Asia and Middle East countries, H9N2 LPAI became endemic since the 1990s (Brown et al. 2006; Sorrell et al. 2007). Highly and low pathogenic H7 influenza have affected the poultry production in many countries including Netherland (Munster et al. 2007), Pakistan (Aamir et al. 2009), and Canada (Berhane et al. 2009). Turkey producers had the biggest challenges with LPAI from avian species in states situated on migratory waterfowl flyways, such as

Minnesota and California (Halvorson et al. 1985). In addition, swine viruses of H1N1, H1N2, and H3N2 subtypes have been a big threat to turkey production since the 1980s (Yassine et al. 2007; Mohan et al. 1981; Suarez et al. 2002).

3 Influenza Transmission Between Pigs and Turkeys

Turkeys are highly susceptible to infection with avian and mammalian influenza viruses (Pillai et al. 2010). Our laboratory was the first to report on the transmission of swine influenza virus to turkeys in the State of Ohio in 1980. Infected turkeys in two flocks experienced decline in egg production and increased percentage of abnormal eggs (Mohan et al. 1981). Serologic evidence indicated a direct transmission of the virus from pigs that were raised in barns of close proximity to turkey houses, and which exhibited influenza-like illness prior to the onset of signs in turkeys.

Since then, cH1N1 swine viruses were frequently isolated from turkey breeder hens in various states in the U.S. which prompted turkey producers to vaccinate their breeder hens with swine influenza viruses circulating in the field (Yassine et al. 2008; Hinshaw et al. 1983; Wright et al. 1992). Similarly, swine influenza viruses were isolated from turkey flocks experiencing drop in egg production in Europe. Based on sequencing analysis of the HA gene, Wood et al. suggested the transmission of swine "avian-like" H1N1 virus from pigs to turkeys in Germany (Wood et al. 1997).

In 2002, Suarez et al. reported the isolation of new swine influenza subtype from turkey flocks in Missouri (Suarez et al. 2002). The virus was characterized as a TR H1N2 virus like those that were previously isolated from pigs in the U.S. Interestingly, the virus was not isolated using the typical chorioallantoic sac route but rather with yolk sac inoculation of embryonated chicken eggs (ECE). Couple of years later, two studies consecutively reported on another new swine influenza isolation from turkey breeder hens in different geographical regions in the U.S. Infections were associated with drastic or complete drop in egg production, with or without other clinical signs including depression, coughing, sneezing, and loss of appetite (Choi et al. 2004; Tang et al. 2005). Viruses were subtyped as H3N2 and characterized as triple TR like those circulating in the swine population since 1998 (Olsen 2002). The virus in this case was not isolated in ECE but after several passages in Madin-Darby Canine Kidney (MDCK) cells. Soon after, we isolated another TR H3N2 virus from a turkey farm in Illinois, in which the turkey hens were vaccinated twice against H3 subtype using swine and avian lineage viruses (Yassine et al. 2008). Such findings prompted us to evaluate the genetic and antigenic relatedness of TR viruses from swine and turkey origin and to study their transmission between the two species.

Although swine and turkey viruses were TR (Cluster III subtype) sharing 95% amino acids sequence identity in most of the genes, they were antigenically distantly related with $< 30\%$ antigenic similarity as evaluated with Archetti-Horsfaul

formula using hemagglutination inhibition (HI) and virus neutralization (VN) tests. We observed at least eight amino acid changes at the main antigenic sites of the HA molecule, which could account for the antigenic difference between the viruses of different lineages (Yassine et al. 2008). In agreement with the above observations, Kapczynski et al. (2009) showed in an experimental study that only viruses highly matched to the field strains would protect from losses in egg production upon turkey infection with TR H3N2 viruses (Kapczynski et al. 2009).

To further understand the TR H3N2 viruses' behavior, we employed several viruses of swine and turkey origin in a comprehensive transmission study between both species (Yassine et al. 2007). We also included cH1N1 and rH1N1 viruses in the transmission experiments for comparison. Neither of the H1N1 viruses transmitted from pigs to turkeys, although the cH1N1 virus manifested the highest replication titer in pigs among all the viruses employed in the study (Yassine et al. 2007). Although turkey H3N2 viruses shared high genetic and antigenic similarity (>99%), they behaved differently in terms of the transmission between pigs and turkeys. Only one virus (A/turkey/Ohio/313053/04) transmitted both ways between the two species, while A/turkey/North Carolina/03 transmitted one way from pigs to turkeys, and A/Turkey/Illinois/04 did not transmit either way between species (Yassine et al. 2007). In brief, our findings indicated the ability of certain H3N2 viruses to transmit between pigs and turkeys, thus, explaining field observations. Despite their difference in transmission behavior, all viruses replicated in pigs and turkeys although to different extents. Additionally, the transmissible strain between swine and turkeys, A/turkey/Ohio/313053/04, replicated and transmitted well among turkeys, replicated and did not transmit among chickens and did not replicate in ducks.

Interestingly, we observed changes in the HA but not the NA protein upon virus transmission between both species. One change was located at the receptor binding domain (RBD) of the HA molecule (Asp190Ala) that occurred upon virus transmission from turkeys to pigs, and another change was observed at residue 246 (Ser246Asn) in two of the inoculated pigs and one of the turkeys in contact with inoculated pigs.

After its emergence in humans in April of 2009, the swine-origin pandemic H1N1 (pH1N1) virus was isolated from pigs and turkeys in different countries in the Americas and Europe (Ariel Pereda et al. 2010; AVMA 2010; Swayne et al. 2009). Initially, experimental infections failed to generate disease in turkeys when using natural route of infection (Terregino et al. 2009; Russell et al. 2009), however, increased susceptibility was demonstrated upon intrauterine inoculation route (Pantin-Jackwood et al. 2010). In our laboratory, we infected juvenile turkeys and adult turkey hens with swine rH1N1 viruses currently circulating in pig population in the U.S. Although we could not detect the virus using real-time RT-PCR (rRT-PCR) in both age groups, both groups seroconverted after two weeks post inoculation. While all turkey hens serocoverted to moderate HI titers (average of 183 HI), five out of six inoculated juvenile turkeys seroconverted to a low HI-titer of 24 (unpublished data).

In summary, turkeys are highly susceptible to infection with swine influenza viruses, however, outcomes of such infections seem to be strain specific. Age of turkeys, their physiological conditions especially at the onset of egg production, their immune status and many other factors at host-virus interaction, seem to affect the susceptibility of turkeys to swine viruses. Currently, autogenous vaccines have been proven to be effective in protecting turkey breeder hens from infections with swine influenza viruses. Continuous surveillance of new subtypes of influenza in pigs and turkeys is very crucial to control the disease in both species, taking into consideration that both animals can be infected with influenza viruses with hidden or non-observed clinical symptoms.

4 Influenza Transmission Between Pigs and Chickens

Chickens are less susceptible to infection with swine influenza viruses compared to turkeys. The cH1N1, TR H1N2, and TR H3N2 influenza viruses have been frequently isolated from turkey hens in North America. Additionally, swine-like TR H3N2 and H1N2 were isolated from waterfowls in the U.S. in two separate occasions (Ramakrishnan et al. 2010; Olsen et al. 2003). Nonetheless, none of these viruses have been isolated from chickens.

In previous work by our group, we inoculated chickens with a swine-origin TR H3N2 influenza virus, A/Turkey/Ohio/313053/04, and placed non-infected control chickens in the same cage at one day post inoculation (DPI) to evaluate virus transmission. The virus replicated to a higher titer in inoculated chickens compared to turkeys, with 60% seroconversion of the infected chickens at 14 DPI (average of 214 HI). However, the virus did not transmit to contact chickens as evaluated with rRT-PCR and HI tests (Yassine et al. 2007). In another study by Thomas et al. (2008), two chicken breeds, white leghorn (WL) and white plymouth rock (WPR), were inoculated intravenously, intranasally, or fed meat contaminated with swine-origin TR H3N2 influenza virus, A/swine/NC/307408/04. None of the infected chickens developed clinical signs and the virus replicated to a very low titer in both chicken breeds. Replication of the virus in both groups was confirmed with a low HI-titer of sera collected at 14DPI. Interestingly, the virus was isolated from swab collected from WL but not WPR, suggesting the higher susceptibility of WL to influenza infection as was previously indicated (Swayne et al. 1994). On the other hand, European swine-lineage H3N2 viruses failed to replicate in SPF WL-chickens in an experimental setup using different routes of inoculations (Campitelli et al. 2002).

Taken together, cumulative results from the above experiments indicate that chickens are less susceptible to infection with swine influenza viruses compared to turkeys. Viruses in all three studies were of the same subtype (H3N2), but were of different origins (swine versus turkeys) or lineages (North American versus European). This could in part explain the variability in the obtained results. Moreover, we have noticed from our experimental infection studies that viruses of

high genetic and antigenic similarity could behave differently in terms of replication and pathogenicity in a specific host (Yassine et al. 2007).

On the other hand, serological and epidemiological evidences indicated the infection of pigs with avian-H4, H5, and H9 subtypes, where the latter two are widely spread in poultry populations in Asia and other continents (Ninomiya et al. 2002). In 2004, Xu et al. reported on reassortments between chicken H5N1 and H9N2 subtypes of chicken or duck origin, resulting in a virus that cause disease and death in pigs (Xu et al. 2004). In another study, phylogenetic analysis on pig-H9 isolates indicated their possible transmission from chickens, where such viruses resulted in respiratory syndrome in pigs, accompanied with coughing, ocular discharge, gored skin, and high fever (Cong et al. 2008, 2007). Interestingly, five internal genes of the H9N2 isolates were highly similar to those of H5N1 lineage viruses cocirculating in pig population in China (Cong et al. 2007).

The HPAI H5N1 viruses emerged in poultry in Asia since 1998 and then spread to many countries around the globe, dramatically affecting the poultry production. The first isolation of the virus from pigs was reported in China in 2004 (Liu et al. 2009). A sero-epidemiological study of pigs in Vietnam in 2004 indicated that only 0.25% (8 of 3175) are positive for H5-subtype antibodies (Choi et al. 2005). Additionally, no seropositive samples were detected in pigs in Korea in a study conducted around the same time (Jung et al. 2007). In Egypt, pigs from 11 herds ($n = 240$) were tested for infection with HPAI-H5N1 viruses using serology and virus isolation (El-Sayed et al. 2010). Serologic testing indicated that 4.6% of the pigs are positive for H5-subtype antibodies, however, attempts failed to isolate the virus from any of the collected samples.

Cumulatively, the above findings suggest the low susceptibility of pigs to infection with HPAI-H5N1 viruses. Supporting these observations, Lipatov et al. (2008) reported that domesticated pigs have low susceptibility to infection with HPAI-H5N1 viruses under experimental conditions. In their study, pigs were inoculated with live viruses or fed meat contaminated with HPAI-H5N1 viruses of human, swan, duck, or chicken origins. None of the viruses caused major illness, and their replication was restricted to the upper respiratory tract, unlike the systemic infection recognized in poultry. Although H5N1 viruses replicated in the respiratory tract of inoculated pigs, their titers in nasal washes were low compared to swine-origin H1N1 and H3N2 viruses used in the same study (Lipatov et al. 2008). Moreover, in an earlier study by Choi et al. (2005) it was clearly shown that pigs can be infected with HPAI H5N1, but these viruses were not readily transmitted between pigs under experimental conditions (Choi et al. 2005).

In addition to the above, pigs were shown to be infected with H7 subtype viruses. In the 2003 influenza outbreak in the Netherlands, pigs were reported positive for H7 antibodies (de Jong et al. 2009; Loeffen et al. 2004). The virus was not isolated from any of the seropositive pigs which were housed in close proximity to poultry infected with the virus. It was suggested that the pigs were infected upon direct exposure to the virus from poultry, with no further transmission amongst pigs. Such conclusion was verified by experimental infection of

pigs with the virus that did not transmit to the contact non-inoculated pigs housed in the same room (de Jong et al. 2009; Loeffen et al. 2004).

5 Determinants for Influenza Transmission Between Pigs and Poultry

The first requirement for influenza virus to infect a new host is the presence of its appropriate receptors on the host cell. Several studies have shown that the pig respiratory tract expresses both avian-like N-acetylneuraminic acid-α2,3-galactose (α2,3Gal) form of sialic acid (SA) receptors as well as human-like α2,6Gal SA receptors, which make them prone to infection with both human and avian influenza viruses (Ito et al. 1998).

Using linkage-specific lectins, like *Maackia amurensis* (MAA, specific for α2-3-linkage) and *Sambucus nigra* (SNA, specific for α2-6-linkage), the distribution of SA receptors in different organs of chickens and turkeys of various age groups was determined (Pillai and Lee 2010). As expected, chickens and turkeys expressed high levels of α2-3 SA receptors in their tracheas. In turkeys, the expression of α2-3 SA receptors was the same in 1-day old, 2–4 week-old, and layer turkeys, while in chickens, the expression in 1-day old was a little bit higher than in the other age groups. Interestingly, both species expressed α2-6 SA receptors in the tracheas of different age groups; however, the expression was more profound in turkeys compared to chickens. While chickens expressed both forms of receptors in the intestines, turkeys exclusively expressed α2-3 SA receptors in the large intestines of all different age groups. The most profound finding was the expression of α2-3 SA receptors in the oviduct of chicken and turkey layers, however, no expression of the same receptors was observed in younger age groups in both species. The latter finding could explain the drastic drop of egg production in turkey hens upon infection with swine viruses (Yassine et al. 2008). It is worth noting that so far, there are no detailed studies that deal with characterization of the receptor binding specificity of TR H3N2 influenza viruses, and hence, no absolute conclusions can be drawn from the above results.

To understand the molecular mechanism(s) behind the transmission of swine influenza to turkeys, we exchanged the two surface glycoprotein (HA and NA) of A/turkey/Ohio/313053/04 that transmitted both ways between swine and turkeys with those of A/swine/North Carolina/03 that did not transmit either way between the two species (Yassine et al. 2007), and evaluated their replication in vitro and in vivo (Yassine et al. 2011). While the exchange of HA and NA genes between the two viruses did not greatly affect their replication in vitro (pig and turkey tracheal epithelial cells), it was clearly indicated that the HA of the turkey strain (A/turkey/Ohio/313053) is crucial for its transmission among turkeys. Nonetheless, HA alone from A/turkey/Ohio/313053/04 in A/swine/North Carolina/03 backbone did not confer the optimum replication of the virus compared to the turkeys strain,

indicating a role of other gene(s) (possibly ribonucleoproteins) in the efficient replication of TR H3N2 viruses in both species.

As was previously mentioned, a mutation at residue 190 of the RBD (Asp190Ala) occurred upon A/turkey/Ohio/313053/04 virus transmission from turkeys to pigs (Yassine et al. 2007). We generated a mutant strain with the above mutation and characterized the virus for its replication in vitro as well as its antigenicity (Yassine et al. 2010). Interestingly, anti-wild type antiserum reacted equally to both wild type and mutant strains, while anti-mutant antiserum exhibited 2–8 fold decrease in the reactivity to the wild type virus. Similar results were obtained when we used a wider range of turkey H3N2 viruses. In addition, the mutation affected virus replication in primary tracheal/bronchial epithelial cells generated from human, pig, and turkey. This was associated with a decrease in binding efficiency to plasma membrane preparations from the three cell types.

In an earlier study, Choi et al. (2004) reported on substitutions at residues 137 and 226 of the receptor binding domain in the turkey H3N2 isolates. Although similar substitutions (Y137S or V226I) were observed in some swine isolates, only turkey viruses had both mutations at the same time. No molecular work was done to study the effect of such mutations on the pathogenicity and antigenicity of the viruses in pigs and turkeys.

6 Concluding Remarks

Despite their significant importance as an intermediate host for influenza virus transmission (Ito et al. 1998), little emphasis has been directed to survey influenza viruses in pigs worldwide. Moreover, modest work has been done to study the interspecies transmission of influenza viruses between pigs and other species, resulting in a lag of understanding the characteristics of these viruses in different animal species. As an example, the pH1N1 that emerged in North America in April of 2009 and rapidly spread around the world is thought to be of swine-origin, although they differ significantly from the currently circulating viruses (rH1N1) in swine populations (Gibbs et al. 2009).

After three decades of reporting the transmission of swine influenza viruses to turkeys (Mohan et al. 1981), we took the initiative to study the transmission of the viruses between both species, trying to unveil the mechanism(s) of such transmission at the molecular level. Results from our laboratory clearly indicated the susceptibility of turkeys to swine influenza viruses, though, the outcomes of turkey infection with such viruses could be very much strain specific. For example, two turkey strains that shared more than 99% of their genome sequences behaved differently in terms of transmission between pigs and turkeys, where one virus (A/turkey/Ohio/313053/04) transmitted both ways between the two species, and the other virus (A/turkey/Illinois/04) did not transmit either way between the two species.

Our preliminary data indicated that the HA gene of TR H3N2 influenza viruses is crucial for virus transmission between turkeys, however, it was not the only player contributing to efficient replication of the viruses in pigs and turkeys. Based on experimental and epidemiological observations, amino acid changes were observed at or close to the RBD upon virus transmission between the two species (Yassine et al. 2007; Choi et al. 2004), however, the characteristic of such mutations and their effect on the receptor binding specificity and pathogenicity is poorly understood. Moreover, no significant work has been done to characterize the receptor binding properties of these viruses after more than a decade of their isolation.

Chickens on the other hand appear to be less susceptible to infection with swine influenza viruses as seen from our laboratory as well as others. Taking into consideration that the virus replicate in chickens even at a low titer, and having in mind the rapid ability of influenza viruses to jump and adapt to a new host, it is important to keep an eye on such viruses in chickens as well as other species.

On the other hand, pigs are also susceptible to infection with influenza viruses of avian origin. Due to the continuous presence of H5N1 and H9N2 viruses in poultry in Asia and Africa, there is always a chance that such viruses might reassort with other mammalian viruses, most probably in pigs, resulting in new strains of unknown threat.

Several studies have shown that the expression of influenza receptors (α2-3 and α2-6 SA) is unique in every host, and a mixture that varies in different organs and different age groups of the same species. Importantly, not only pigs can be considered as "mixing vessels", but also a wide range of other mammalian and avian species. Thus, continuous surveillance of influenza viruses in pigs and poultry is very important to control the disease in both animal species. Additionally, it is necessary to characterize at the molecular level the interspecies transmission of such viruses and to develop better vaccines that can counter heterologous viruses' challenges.

Influenza viruses are not highly resistant and can be inactivated under various physiochemical and thermal conditions. Nevertheless, influenza continues to spread worldwide and tremendous work is needed to reduce the threat of the virus in animal species.

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The 2009 Pandemic Influenza Virus: Where Did It Come from, Where Is It Now, and Where Is It Going?

Ian York and Ruben O. Donis

Abstract Around 2008 or 2009, an influenza A virus that had been circulating undetected in swine entered human population. Unlike most swine influenza infections of humans, this virus established sustained human-to-human transmission, leading to a global pandemic. The virus responsible, 2009 pandemic H1N1 (H1N1pdm), is the result of multiple reassortment events that brought together genomic segments from classical H1N1 swine influenza virus, human seasonal H3N2 influenza virus, North American avian influenza virus, and Eurasian avian-origin swine influenza viruses. Genetically, H1N1pdm possesses a number of unusual features, although the genomic characteristics that permitted sustained human-to-human transmission are yet unclear. Human infection with H1N1pdm has generally resulted in low mortality, although certain subgroups (including pregnant women, people with some chronic medical conditions, morbidly obese individuals, and immunosuppressed people) have significantly higher risk of severe disease. As H1N1pdm has spread throughout the human population it continued to evolve. It has also reentered the swine population as a circulating pathogen, and has been transiently identified in other species such as turkeys, cats, and domestic ferrets. Most genetic changes in H1N1pdm to date have not been clearly linked to changes in antigenicity, disease severity, antiviral drug resistance, or transmission efficiency. However, the rapid evolution rate characteristic of influenza viruses suggests that changes in antigenicity are inevitable in future years. Experience with this first pandemic of twenty-first century reemphasizes the importance of influenza surveillance in animals as well as humans, and offers lessons to develop and enhance our ability to identify potentially pandemic influenza viruses in the future.

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Current Topics in Microbiology and Immunology (2013) 370: 241–257

DOI: 10.1007/82_2012_221

Published Online: 26 May 2012

Contents

1 Introduction

Sometime between mid-2008 and early 2009 (Smith et al. 2009), a new influenza A virus from animals infected humans and established sustained human-to-human transmission chains. After circulating in Mexico for a number of weeks, influenza surveillance of humans identified this as a novel virus of swine origin in April of 2009 (2009b; Echevarria-Zuno et al. 2009), by then hundreds of thousands of people may have already been infected. Shortly thereafter the virus, variously called as swine flu, swine-origin influenza virus (SOIV), or 2009 pandemic H1N1 (H1N1pdm, the term used in this review), rapidly spread throughout the world as the first pandemic influenza in over 30 years.

2 Where Did It Come from?

Although H1N1pdm of humans clearly arose from an influenza virus of swine, its evolutionary history is still incompletely understood due to lack of fossil data from virologic surveillance (Garten et al. 2009; Smith et al. 2009). This virus contains segments of both North American and Eurasian swine influenza virus (SIV) strains, in a combination that has not been described previously (Garten et al. 2009; Smith et al. 2009). These genes contributed by the North American swine influenza virus were traced to so-called "triple reassortant" viruses, which emerged in 1998 and have circulated without interruption in swine till date. The reassortment that gave rise to the 1998 swine virus involved the following: (1) "classical" H1N1 influenza viruses of swine, derived from the 1918 H1N1 human pandemic virus or from a shared ancestor (Gorman et al. 1991); (2) a contemporary human H3N2 virus; and (3) avian viruses circulating in North America. The "triple-reassortant" (TR) swine H3N2 viruses acquired HA, NA, and PB1 genes from the human influenza virus, and PB2 and PA from the avian virus with remaining genes from the classical swine influenza virus parent (Karasin et al. 2000; Webby et al. 2000; Zhou et al. 1999). However, further reassortment of the H3N2 TR virus in early 2000 led to the exchange of the H3N2

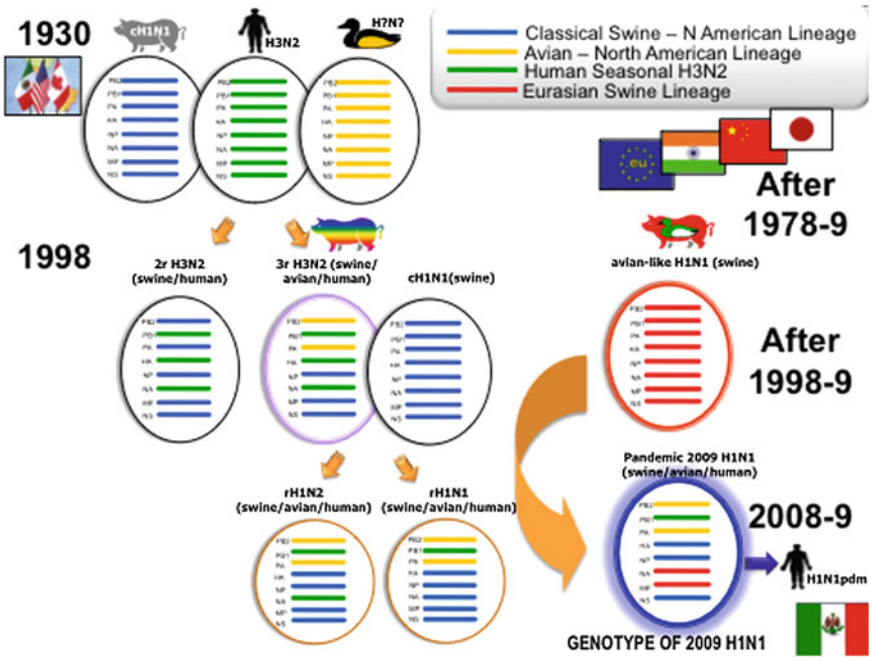

Fig. 1 Genetic origins of H1N1pdm. See text for details

HA and NA genes with the classical swine H1N1, with retention of PB1 from the human donor virus and PB2 and PA from the avian influenza virus (Webby et al. 2004); these viruses were designated TR swine H1N1. Meanwhile, in Eurasia, an influenza virus from birds was transmitted to swine in the 1970s and has circulated in swine since (Brown 2000; Maldonado et al. 2006; Pensaert et al. 1981). The TR H1N1 swine influenza virus acquired the NA and M genes from Eurasian swine influenza viruses, generating a novel reassortant virus that was the parent of the pandemic H1N1 virus (Garten et al. 2009; Smith et al. 2009) (Fig. 1). This novel virus may have circulated in one or more animal hosts for several years before its emergence among humans, but was not detected due to the paucity of global influenza surveillance of swine.

The classical swine H1N1 viruses from the modern virology era (since approximately the late 1950s) are considered to be less well adapted to humans than to swine, because although they occasionally have infected humans, these cases have failed to establish sustained transmission (Myers et al. 2007; Thacker and Janke 2008). Similarly, the TR SIV subtype H1N1 caused sporadic zoonotic infections in humans without any evidence of further human-to-human transmission (Myers et al. 2007; Shinde et al. 2009). By contrast, the 2009 pandemic H1N1 virus was able to establish sustained transmission in the human population. What characteristics of the virus enabled this new ability?

A number of groups have developed algorithms to help distinguish influenza viruses with potential human pathogenicity and/or high virulence from those that are restricted to avian or other species (Allen et al. 2009; Chen and Shih 2009; Taubenberger et al. 2005). In general, these approaches have not been highly predictive for viruses of swine origin. For example, out of 47 amino acids identified as avian-human signatures, 36 of them show avian-like patterns in H1N1pdm, and only one of the "signature" amino acids differs between human-adapted H1N1pdm and its swine-specific ancestors (Chen and Shih 2009).

One possibility is that the NA and M genes of the Eurasian "avian-like swine H1N1" viruses provided some unique functional properties when acting in concert with the genes from the triple reassortant swine H1N1 viruses. A number of genetic features of H1N1pdm have been assessed for their role in infecting humans (Table 1).

Influenza RNA-dependent RNA polymerase, comprising PB1, PB2, and PA, is believed to be important for adaptation of avian influenza viruses by human and other mammalian hosts (Li et al. 2009). In particular, the PB2 protein often influences replication capacity of certain avian influenza viruses in mammalian versus avian cells. Several mutations in PB2, including E627K, D701N, E677G, and T271A, generally enhance polymerase activity in mammalian cells. The PB2 of H1N1pdm retains avian-like sequence features, but after more than a decade of circulation in swine, it would be expected to be optimized for replication in mammalian cells nonetheless. However, PB2 protein of H1N1pdm does contain a T271A mutation, which enhances replication and growth in mammalian cells (Bussey et al. 2010). Although PB2 from H1N1pdm lacks E627K, D701N, and E677G mutations associated with efficient avian influenza infection of human cells (Garten et al. 2009), compensating mutations, particularly a basic residue at position 591 and a mutation to S at position 590, permit efficient human infection (Mehle and Doudna 2009; Yamada et al. 2010). Experimental mutation of H1N1pdm E627 to K, D701 to N, or E677 to G does not enhance H1N1pdm replication in human cells or pathogenesis in animals (Herfst et al. 2010; Jagger et al. 2010; Zhu et al. 2010), although these changes have been linked to avian influenza virulence in mammals.

The presence of a full-length PB1-F2 protein has been correlated with influenza virus virulence in mice (Conenello et al. 2007). In H1N1pdm, PB1-F2 is truncated due to the presence of premature stop codons (Garten et al. 2009; Smith et al. 2009). However, mutating the H1N1pdm version of PB1-F2 to restore expression of full-length proteins, including a version (with N66S substitution) previously linked to increased viral virulence (Conenello et al. 2007), did not alter the virulence of H1N1pdm in mice or ferrets (Hai et al. 2010).

NS1 is a multifunctional protein that, among other functions, prevents innate immune recognition of viral RNA and/or activation of inflammatory pathways (Ehrhardt et al. 2010; Hale et al. 2008). In H1N1pdm, NS1 is truncated at the C terminus resulting in loss of the PDZ binding (Hale et al. 2010; Jackson et al. 2008), and contains a mutation (K217E) that prevents it from binding to the Crk/CrkL signaling adapters (Garten et al. 2009). However, reversion of these changes to the sequence of seasonal influenza's NS1 did not significantly alter

Table 1 Genetic variations of pandemic H1N1 influenza virus

Variations identified in original isolates			
Protein	Variant	Presence in H1N1pdm genome	Phenotype
PB2	E627K[a, b]	Not present	Enhance replication in mammalian cells
	T271A[a, b]	Not present	Enhance replication in mammalian cells
	590/591 SR[c]	Present	Permits efficient human infection
	D701N[d]	Not present	Increased viral virulence
	E677G[d]	Not present	Increased viral virulence
PB1-F2	Stop codons[e]	Present	No full-length PB1-F2
	N66S[e]	Not present	Increased viral virulence
NS1	C-terminal truncation[c, f]	Present	Loss of PDZ binding domain
	K217E[a, b]	Present	Prevent binding to Crk/CrkL signaling adapters
M2	S31N[a, b]	Present	Adamantane resistance
Mutations identified in natural infections during the pandemic			
Protein	Variant	Viral condition	Phenotype
HA	D222G[g–n]	Sporadic	Increased virulence?
N	H275Y[o–s]	Sporadic	Osteltamivir resistance
PB2	K340N[t]	Sporadic	Unknown
HA	E391K[t]	Increasing	Unknown
HA	S206T[u]	Increasing	Unknown
NP	V100I[u]	Increasing	Unknown
NA	V91I[u]	Increasing	Unknown
NA	N233D[u]	Increasing	Unknown
NS1	I123V[u]	Increasing	Unknown

The genome of H1N1pdm contained a number of variant positions that differed from other strains of influenza and that in some cases had been linked with phenotypic changes. Other mutations arose either sporadically, or in increasing numbers, as the pandemic progressed

References [a] (Garten et al. 2009), [b] (Smith et al. 2009), [c] (Mehle and Doudna 2009), [d] (Herfst et al. 2010), [e] (Conenello et al. 2007), [f] (Hale et al. 2010), [g] (Shinya et al. 2006), [h] (Stevens et al. 2006b), [i] (Kilander et al. 2010), [j] (Mak et al. 2010), [k] (Melidou et al. 2010), [l] (Miller et al. 2010), [m] (Puzelli et al. 2010), [n] (2010b), [o] (Esposito et al. 2010), [p] (Harvala et al. 2010), [q] (Memoli et al. 2010), [r] (Wang et al. 2010), [s] (Zepeda et al. 2010), [t] (Maurer-Stroh et al. 2010), [u] (Pan et al. 2010)

H1N1pdm's replication or virulence in cells or in animals (Hale et al. 2010). This may be related to the observation that H1N1pdm infection of cells induces a weaker cytokine response than seasonal influenza (Osterlund et al. 2010), so potentially there is less requirement for NS1 inhibition of cytokine responses. Similarly, although NS1 from H1N1pdm was less effective at blocking innate immune responses in cultured cells, modifying H1N1pdm NS1 to more closely resemble that to the seasonal strains of influenza reduced the virulence of H1N1pdm and allowed it to be more rapidly cleared, even though the modified protein was able to more effectively antagonize innate immunity (Hale et al. 2010).

A critical characteristic of H1N1pdm is that it is antigenically distinct from seasonal H1N1 of recent years (Garten et al. 2009). Sera that neutralize recent seasonal H1N1 strains do not neutralize H1N1pdm, and therefore recent exposure to influenza viruses, or vaccination against influenza viruses, did not confer protection against H1N1pdm. Only among people over the age of about 60 years has immunity toward H1N1pdm common (Hancock et al. 2009), indicating that an antigenically similar virus probably circulated as a seasonal influenza virus in the 1940s or 1950s but was then displaced by antigenically different strains. This virus was therefore able to spread throughout the human population without encountering large numbers of immunologically resistant people.

It is important to note that although antigenic novelty allowed H1N1pdm to spread between naive hosts, other swine influenza viruses are also antigenically novel, and in spite of widespread contact between people and swine, those other viruses were not able to establish efficient human-to-human transmission. In general, then, it remains unclear which genetic changes in H1N1pdm, compared to its multiple ancestors, allowed this virus to efficiently infect and transmit among humans.

3 Where Is It Now?

Pandemic H1N1 first appeared in Mexico in or around February 2009 (2009b; Garten et al. 2009; Smith et al. 2009). It was identified as a novel strain in April 2009, and rapidly spread throughout the world in next few weeks. In fact, H1N1pdm may have already spread to Australia even before its identification in the United States (Kelly et al. 2010). H1N1pdm Infection was widespread in the temperate latitudes of the Southern Hemisphere during May to September, peaking in June to August (Baker et al. 2009), but was sporadic in the Northern Hemisphere, approximating the pattern for influenza infection in each hemisphere (http://www.cdc.gov/flu/weekly/, accessed July 2010). In the Northern Hemisphere a new, large wave of infections recurred in fall, peaking around October/November of 2009, significantly earlier than the usual peak of seasonal influenza infections (http://www.cdc.gov/flu/weekly/, accessed July 2010). In comparison to the 1918/1919 and 1957/1958 pandemics suggested that there might have been another wave in the Northern Hemisphere winter and spring of 2010, but this

anticipated winter wave did not occur. By winter of 2010, immunity toward the virus as a result of infection (about 60 million people), vaccination (over 70 million people), and preexisting immunity to H1N1pdm (over 25 million people) in the US population approached 50 % of the population, with immunity in the most susceptible populations (the young and the old) being significantly higher than the average (2010a). In a community-based study, vaccine coverage of 80 % was sufficient to markedly reduce community infection with influenza virus (Loeb et al. 2010), and it is possible that the expected winter wave of infection was forestalled by preexisting immunity toward H1N1pdm and toward seasonal H1N1.

In humans, H1N1pdm infection has been characterized by relatively low virulence and transmissibility as compared to the previous seasonal human influenza viruses. Mortality rates were estimated at about 0.05 % (Donaldson et al. 2009; Hadler et al. 2010; Kamigaki and Oshitani 2009; Presanis et al. 2009), similar to or even lower than normal seasonal influenza mortality rates. Groups at particular risk of severe disease or death include those with chronic medical conditions, such as diabetes or respiratory conditions (Jain et al. 2009; Kumar et al. 2009; Nguyen-Van-Tam et al. 2010); pregnant women (Fuhrman et al. 2010; Jain et al. 2009; Vaillant et al. 2009); obese and especially morbidly obese individuals (Fuhrman et al. 2010; Jain et al. 2009; Morgan et al. 2010; Nguyen-Van-Tam et al. 2010; Vaillant et al. 2009); and immunosuppressed and immunodeficient patients (Fuhrman et al. 2010; Gordon et al. 2010).

Transmissibility (R_0) for H1N1pdm is estimated to be around 1.3–1.7 (Balcan et al. 2009; Tuite et al. 2010; Yang et al. 2009), which is also similar to or lower than estimates for seasonal influenza (Chowell et al. 2007, 2008; Coburn et al. 2009; Truscott et al. 2009) and lower than those for previous influenza pandemics (Chowell et al. 2007; Coburn et al. 2009; Sertsou et al. 2006).

Inference of the intrinsic virulence of the virus from epidemiologic data is complicated due to variable levels of protective immunity toward the virus in the human population. In particular, adults over age of 60 (who are usually the highest risk group for complications of influenza) were infected by H1N1pdm only relatively rarely, most likely due to protective immunity established by exposure to related strains of virus in the 1950s and earlier (Lemaitre and Carrat 2010; McLean et al. 2010; Skountzou et al. 2010; Viboud et al. 2010; Xu et al. 2010), so that the virus spread mainly among younger people. As a result of this preferential infection of younger people and in spite of the relatively low case-fatality rate, the 2009–2010 H1N1 pandemic was estimated to cause between 334,000 and 1,973,000 years of life lost in the US alone (Viboud et al. 2010).

Early in the course of the pandemic, the characteristics of the new virus were unclear. To understand and predict the course of the pandemic, the virus was analyzed in tissue culture and in experimental animals. The HA of H1N1pdm binds specifically to α2–6 sialosides (Childs et al. 2009; Maines et al. 2009; Yang et al. 2010), a human receptor specificity like those of normal human seasonal influenza strains, although some weaker binding to α2–3 sialosides have also been described (Childs et al. 2009). The cell tropism of H1N1pdm was very similar to the seasonal strains (Chan et al. 2010). Mice infected with H1N1pdm developed mild to moderate

pathology, generally similar to seasonal strains (Belser et al. 2010). Ferrets infected with H1N1pdm showed somewhat more severe symptoms and pathology than those infected with seasonal H1N1 strains (Itoh et al. 2009; Maines et al. 2009; Munster et al. 2009; van den Brand et al. 2010). Different groups found conflicting results on droplet transmission between ferrets, determining that that transmission was less efficient than (Maines et al. 2009), similar to (Munster et al. 2009), or more efficient than (Perez et al. 2009) that of seasonal H1N1.

As commonly found in Eurasian SIV (Krumbholz et al. 2009), H1N1pdm contains an S31N variation in the M2 protein that confers resistance toward the adamantane antivirals (Garten et al. 2009), and virtually all H1N1pdm isolates were adamantane resistant (2009c). However, initial viral isolates were susceptible to the neuraminidase inhibitor oseltamivir (Garten et al. 2009; Smith et al. 2009), and only sporadic instances of oseltamivir resistance have been observed since (see below).

4 Where Is It Going?

It is likely, though not certain, that the H1N1pdm will establish itself long-term in humans, with patterns of transmission similar to those of the previous seasonal influenza viruses. Although extinction of this virus is possible, seroepidemiologic studies suggest that immunity in the community remains near 50 %, and therefore, the virus is likely to remain in circulation.

Historically, the emergence of pandemic influenza viruses has usually (but not always) coincided with extinction of the previously circulating virus and widespread circulation of the pandemic influenza virus in subsequent years. This replacement, though not well understood, is probably attributable to temporary cross-reactive immunity, probably mainly through T cells (Greenbaum et al. 2009; Pascua et al. 2009; Steel et al. 2010), and/or because the pandemic virus has higher fitness than the seasonal strains (Perez et al. 2009). In the 2009–2010 season, this viral replacement may have been aided by unusually high uptake of seasonal influenza vaccines (2010a), leading to the widespread immunity against seasonal influenza. On the other hand, the relatively low H1N1pdm infection rate in older people (Fisman et al. 2009; Hancock et al. 2009; Kamigaki and Oshitani 2009; Mu et al. 2010; Shen and Lu 2010) may have resulted in a population that did not develop the transient cross-reactive protection and that remained susceptible to seasonal H1N1 and H3N2 strains. Similarly, since killed vaccines are better inducers of antibody than of the more cross-reactive T cell immune responses, the tens of millions of individuals who were vaccinated against H1N1pdm may also provide a population in which seasonal viruses could persist.

At the time of writing, virologic surveillance of circulating viruses has not conclusively determined which viruses will persist into and past the 2010/2011 influenza seasons. Although H3N2 and seasonal H1N1 infections were still detected through the 2009–2010 influenza season, these viruses were apparently relatively

rare; more than 99 % of subtyped influenza viruses in the US were H1N1pdm. H3N2 strains were identified more often than seasonal H1N1, suggesting that H1N1pdm infections may more effectively interfere with the cocirculation of the other H1N1 viruses than with H3N2. The WHO vaccine composition recommendations for 2010–2011, therefore, include an H3N2 component as well as an influenza B, but did not include a seasonal H1N1 (2010c). If the seasonal H1N1 strain as well as H1N1pdm and H3N2 were to persist, it might be necessary to use a quadrivalent vaccine (H1N1pdm, H3N2, seasonal H1N1, and B) to provide protection against all the circulating influenza strains in coming years.

4.1 Assessing and Monitoring Viral Virulence and Mutations

The relatively recent establishment of the H1N1pdm virus in the human population could be considered insufficient for complete adaptation to humans. Thus, a major concern over H1N1pdm remains is its potential to become more virulent or to acquire antiviral drug resistance, either due to reassortment with other influenza strains, or through mutations. Coinfection of individuals with multiple different influenza strains is not unusual, occurring in between 0.5 and 3 % or more cases (Ghedin et al. 2009), and coinfection by H1N1pdm and seasonal H3N2 has been reported (Lee et al. 2010), supporting concerns about reassortment between strains. However, the low frequency of seasonal influenza strains in 2009–2010 makes reassortment in humans relatively unlikely. If other strains of influenza A become more widespread and cocirculate with H1N1pdm, this may increase the probability of reassortment. Experimental reassortants between H1N1pdm and highly pathogenic H5N1 avian influenza viruses were found to be replication competent and in some cases grew more rapidly than their parents, raising the possibility of a pandemic H5N1/H1N1pdm reassortant (Octaviani et al. 2010).

On the other hand, influenza virus is capable of rapid mutation that can provide a substrate for natural selection of strains with altered characteristics. As noted above, although a number of influenza mutations have been previously associated with altered virulence or transmissibility, in general experimental introduction of such mutations into the H1N1pdm genome has not significantly affected its characteristics in cell or animal infections (Hai et al. 2010; Hale et al. 2010; Herfst et al. 2010; Jagger et al. 2010; Zhu et al. 2010). Therefore, it will be important to correlate genetic changes with epidemiologic data from H1N1pdm infections (Table 1).

At least seven geographically dispersed clades of H1N1pdm viruses were detected after their initial global spread (Nelson et al. 2009), and the divergence has been continuous (Melidou et al. 2010). No clear difference in viral pathogenicity, fitness, or drug resistance has been linked to the different clades yet. However, a number of mutations have been linked to differences in viral characteristics, in particular the D222G mutation in HA that may be associated with severe disease, and the H275Y mutation in N associated with oseltamivir resistance. In addition,

a K340N mutation in PB2 has also been detected in some patients with severe disease (2010b; Maurer-Stroh et al. 2010), but it is not clear whether this mutation itself alters viral characteristics, whether it is a passenger on some other change that does alter viral virulence, or indeed whether the apparent association with virulence is a real one (2010b).

Mutation of the HA D222 to G has been associated with a change in receptor specificity that may allow viruses with this change [possibly including H1N1pdm (2010b, d)] to more efficiently infect the lower respiratory tract (Shinya et al. 2006; Stevens et al. 2006a). A number of reports have linked D222G-containing H1N1pdm viruses with more severe infections (Kilander et al. 2010; Mak et al. 2010; Melidou et al. 2010; Miller et al. 2010), although the clinical relevance of this change remains unclear (2010b). Although D222G-containing H1N1pdm viruses have been identified in circulation (Melidou et al. 2010; Puzelli et al. 2010), and this variant has been associated with increased severity of disease in mice (Ilyushina et al. 2010; Zheng et al. 2010) the change does not seem to be associated with significantly enhanced viral fitness, since most of these mutations seem to be sporadic in nature (2010b; Melidou et al. 2010).

Oseltamivir, a neuraminidase inhibitor, was widely used in treatment of H1N1pdm patients with severe symptoms. Resistance to oseltamivir occurs with a single mutation in neuraminidase, H275Y. Although this mutation is associated with reduced viral fitness (Herlocher et al. 2004; Ives et al. 2002), compensating mutations (Bloom et al. 2010) allowed oseltamivir-resistant seasonal influenza bearing this mutation to spread globally in 2008 (Moscona 2009). Similarly, oseltamivir-resistant H1N1pdm are as virulent as are wild-type H1N1pdm in mice and ferrets (Hamelin et al. 2010). Development of oseltamivir resistance is of particular concern in immunocompromised patients, who may receive relatively long-term treatment with oseltamivir, allowing the development of resistant virus (2009a; Moscona 2009; Tramontana et al. 2010). In the case of H1N1pdm, although multiple cases of oseltamivir resistance have been described, to date all have been sporadic in nature and have shown little or no human-to-human spread (Esposito et al. 2010; Harvala et al. 2010; Memoli et al. 2010; Wang et al. 2010; Zepeda et al. 2010).

As well as these mutations that have been (at least tentatively) linked to changes in viral characteristics, some mutations have increased in frequency over time. These include changes in NP (V100I), NA (V91I and N233D), HA (E391K and S206T), and NS1 (I123V) (Table 1) (2010b; Maurer-Stroh et al. 2010; Pan et al. 2010). The functional and clinical significance (if any) of these changes remains unknown.

Antigenic drift is a common event in seasonal influenza viruses. As immunity to currently circulating H1N1pdm becomes widespread in the population, mutations in antigenic sites of the HA that confer increased ability to evade humoral immunity can rapidly spread throughout the newly susceptible population. Although antigenic drift *per se* does not alter viral virulence, if other viral mutations that do confer altered virulence are linked to the new HA variant, such changes can in turn spread extensively. At the time of writing, although mutant H1N1pdm viruses with altered antigenicity have occasionally been detected,

they have not spread widely; the vast majority of H1N1pdm isolates have been antigenically closely related to the earliest isolates from which vaccines were developed. This may be because there has not been enough population immunity to confer a selective advantage to antigenic variants as yet.

In terms of population immunity, the H1N1pdm pandemic may be most similar to the 1976/1977 reintroduction of H1N1 into a human population from which it had been absent for nearly 20 years, since in both cases younger people were susceptible while older people were often immune. In that case, the virus remained relatively stable antigenically for several years: Not until 1983 strains that were significantly different antigenically become widespread.

4.2 Potential for Interspecies Transmission and Reassortment

Several species could support coinfections with H1N1pdm and other influenza virus leading to reassortment. Pandemic H1N1 has been shown to infect multiple species other than humans. Sporadic infection of domestic cats (Lohr et al. 2010; Sponseller et al. 2010), ferrets (Patterson et al. 2009), dogs (http://www.avma.org/public_health/influenza/new_virus/default.asp accessed July 2010), and other animal species (Bao et al. 2010; Britton et al. 2010) has been reported. Of particular concern is infection of species that are routinely infected with other strains of influenza, such as swine and birds, since infection of these animals has the potential to generate reassortants.

Unsurprisingly, considering the origin of the virus, H1N1pdm readily infects swine (Itoh et al. 2009; Lange et al. 2009; Weingartl et al. 2010), and several natural infections of swine herds have been reported (Hofshagen et al. 2009; Howden et al. 2009; Pasma and Joseph 2010; Welsh et al. 2010) (http://www.oie.int/wahis/public.php?page=single_report&pop=1&reportid=9151 accessed July 2010). Both experimentally and in natural cases, the disease in swine is mild (Itoh et al. 2009; Lange et al. 2009; Weingartl et al. 2010), and it would therefore not be surprising if reports of infected herds underestimate the actual number of infections. It is possible that H1N1pdm will, or has already, become established in pigs as an enzootic virus. Since multiple strains of influenza virus can infect swine, these represent a potential source of reassorted and mutant viruses that could spread to humans (Bi et al. 2010). Indeed, a reassortant between H1N1pdm and a TR SIV has recently been identified (Vijaykrishna et al. 2010), and more reassortants are likely to arise in coming months and years.

Although turkeys are relatively resistant to H1N1pdm infection experimentally (Kalthoff et al. 2010), natural infection of turkey flocks has also been reported (http://www.oie.int/wahis/public.php?page=single_report&pop=1&reportid=8389, http://www.oie.int/wahis/public.php?page=single_report&pop=1&reportid=8578, http://www.oie.int/wahis/public.php?page=single_report&pop=1&reportid=8709), again with mild symptoms. Ducks and chickens are resistant to experimental infection with H1N1pdm (Bao et al. 2010; Kalthoff et al. 2010).

5 Conclusions

After the 1957/1958 influenza pandemic, Martin Kaplan observed that "Despite the fact that the recent pandemic was the best studied and recorded to date, the knowledge gained will probably have little predictive value for the next pandemic, either in qualitative or quantitative terms" (Kaplan 1960). The 2009 H1N1 pandemic is now the best studied pandemic on record. Is Kaplan's comment still applicable in 2009? Indeed, experience from previous pandemics was not very predictive for the 2009 H1N1 pandemic, since that experience predicted that new pandemic influenza viruses would arise from Asia, rather than North America; that the subtype would not be the same as a circulating seasonal strain; that the immediate mechanism would involve reassortment among different strains; and that the donor of the novel HA would be avian.

Although a new influenza pandemic was considered inevitable, the swine H1N1pdm pandemic still offered a number of surprises and lessons. The genetic features of this virus that permitted efficient human infection and person-to-person transmission remain unclear. The fact that the virus has relatively low virulence was fortuitous, and the features that are associated with low versus high virulence in swine origin influenza viruses remain unclear. Predictions of virulence and transmissibility from genetic findings, mostly based on avian influenza viruses, were not broadly informative. The low level of surveillance of swine forces inference, rather than observation, of the origins and genetic history of this virus. These findings also mean that predicting the future course of H1N1pdm (and other influenza viruses) in the human population will be difficult, since it is difficult to know which mutations have the potential to increase virulence and/or transmissibility.

Epidemiologically, H1N1pdm reminds us that influenza pandemics can spread with explosive speed and are unlikely to be halted or effectively controlled by quarantine. However, the lack of a winter wave of H1N1pdm does hint that population immunity, and therefore rapid deployment of vaccines, may be useful in reducing spread. Experience with seasonal influenza in terms of timing and demographics may not be applicable to pandemic influenzas, and new strategies for vaccine production may be necessary to generate sufficient doses in time.

In conclusion, experience with H1N1pdm should help guide research into the understanding of virulence, transmission, and prevention of influenza in order to rapidly and effectively respond to the inevitable next pandemic influenza outbreak.

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Pandemic Influenza A H1N1 in Swine and Other Animals

Julia Keenliside

Abstract Influenza A virus infection has been reported in a variety of mammalian and avian species. Wild waterfowl such as ducks and geese are considered the principal reservoir of many influenza A viruses. On May 2, 2009, the first confirmed case of pandemic 2009 H1N1 (pH1N1) in animals was reported in a small swine herd in Canada. A public health investigation concluded that transmission from people to pigs was the likely source of infection. Subsequently the pH1N1 virus has been reported in turkeys, cats, dogs, ferrets, and several wildlife species. Human to animal transmission has been confirmed or suspected in a number of cases. The naming of the virus as "swine flu" in the international media led to a drop in the demand for pork and subsequently a reduction in the price of pork paid to farmers. Estimates of losses to pork producers in North America run into hundreds of millions of dollars. Increased surveillance of swine populations for influenza viruses has been suggested as a control measure against the development of future pandemic viruses. In order to be successful, future surveillance and reporting policies must include provisions to protect the livelihoods of farmers.

Contents

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Current Topics in Microbiology and Immunology (2013) 370: 259–271

DOI: 10.1007/82_2012_301

Published Online: 21 December 2012

1 Introduction: Influenza A Viruses in Animals

Influenza A virus infection has been reported in a variety of mammalian and avian species, including horses, dogs, cats, pigs, ferrets, chickens, turkeys, ducks, geese, seals, and mink. Wild waterfowl such as ducks and geese are considered as the principal reservoir of these viruses (Van Reeth 2007).

Influenza virus generally produces mild to moderate symptoms of short duration in mammalian species, with mortality being uncommon. Most mammals exhibit illness consistent with respiratory infection: pyrexia, anorexia, malaise, conjunctivitis, coughing, and sneezing. Pathological lesions are consistent with viral pneumonia. Outbreaks in naive groups of animals can produce morbidities of 100 %. In swine herds infection may result in reduced growth and reproductive performance and increased secondary illness. The severity of symptoms in swine is quite variable, depending on viral subtype, the presence of other respiratory diseases and maternal immunity (either from natural infection or vaccination).

In domestic swine, influenza is a common seasonal infection seen in all major pig producing regions of the world. Three subtypes of influenza A are regularly seen in North America: classical H1N1, H1N2, and H3N2. The classical H1N1 has been known to exist for many decades and has stayed relatively unchanged. It is ancestrally related to the 1918 H1N1 human influenza virus. It has been postulated that this virus jumped from humans to swine in the USA during the pandemic. In 1998, two new triple reassortments appeared in swine in the USA, with human like genes, indicating viral change (Webby et al. 2000). Seasonal outbreaks of influenza in commercial swine farms historically were seen between November and May, but since 1998, the disease has changed expression to occur year round and be more pathogenic (Gramer 2009).

In aquatic birds, the virus typically targets the digestive tract rather than the respiratory tract, thus respiratory symptoms are not seen. Symptoms are highly variable in birds, ranging from mild to severe depending on the virus strain, age, and species of bird (Alexander 2006). H5, H7, and H9 strains are typically seen in domestic avian species. Swine influenza (H3 subtype) infection in domestic turkeys has caused reduced number and quality of eggs but no other symptoms (Choi et al. 2004).

Transmission of influenza between animals is believed to occur mainly through contact with infected respiratory secretions. Contact may be directly with an

infected animal, or by inhalation of aerosolized virus, or by indirect spread via contaminated equipment or clothing. Influenza viral subtypes are usually host species specific and cross species transmission has been considered rare (Van Reeth 2007) but in the past decade cross-species transmission of several influenza virus subtypes has been reported more frequently in the literature. Examples include swine H1N1, H1N2, and H3N2 viruses in turkeys (Choi et al. 2004), a human H1N2 virus in swine (Karasin et al. 2006), and avian viruses in swine (Karasin et al. 2000; Karasin et al. 2004). Avian influenza viruses more frequently circulate between water birds and domestic poultry and infect mammals less frequently. Transmission of an H3N2 swine virus to both turkeys (Yassine et 2007) and a swine worker has been reported (Olsen et al. 2006). Swine viruses have been isolated from humans on a number of occasions and infection in swine workers may be more frequent than previously reported (Myers et al. 2006).

Coinfection with two different influenza viruses could lead to reassortment and the emergence of a novel influenza with potential public health significance. Virus receptors differ between mammals and birds but swine have cell surface receptors for both avian and mammalian viruses. Transmission of avian viruses is therefore easier to swine than humans. As a result, attention has been focused on swine as a potential "mixing vessel". Pigs are often in close contact with poultry, ducks and humans, especially in Asia, where many new virus strains originate (World Health Organization 2009a, b, c, d; Van Reeth 2007).

Since influenza virus infections in most mammals are ubiquitous and generally mild, no public health action is currently taken when outbreaks occur. Vaccination of swine for influenza is common in many pig producing regions to reduce economic losses. Influenza in mammalian species is not reportable and is not monitored by the World Organization for Animal Health (OIE—Office International des Epizootics). In poultry, certain influenza subtypes (e.g., H5, H7) are reportable to the OIE because of their potential to be highly pathogenic to both birds and human (OIE 2012a, b). Regulatory action such as testing, monitoring, and control through depopulation of infected birds is usually undertaken with avian influenza strains.

2 Experimental pH1N1 Infection in Animals

Given the genetic make-up of pH1N1, it was postulated early on the pandemic that swine and a number of other species such as poultry, cats, and dogs could be infected by this virus (Evolution of Pandemic H1N1 2009 in animals—OIE Press Release Nov 4, 2009).

Experimental infection of pigs with the pH1N1 influenza virus A/Regensburg/D6/09 produced mild symptoms of pyrexia, nasal discharge, fever and diarrhea. Viral shedding began at 1 day post infection and the virus was effectively transmitted to na pigs through direct contact (Lange et al. 2009a, b). Other studies with experimental infection of swine with pH1N1 have also produced typical influenza symptoms of short duration (Vincent et al. 2009; Brooks et al. 2009).

Experimental infection of specific pathogen free miniature pigs with an early pH1N1 isolate (A/California/04/09) produced no clinical signs, although viral replication was detected in the respiratory tract and pathological lesions such bronchiolitis were noted (Yasushi et al. 2009). Infection of three other nonhuman species with the same virus produced more severe pathological lesions than other circulating human H1N1 viruses. Clinical illness and bronchitis, bronchiolitis, and aveolitis were seen in 8 month old male domestic ferrets (*Mustela putorius* furo), 3–4 year old cynomologous macaques (*Macaca fascicularis*) and 5 week old female BALB/c mice (*Mus musculus*). In addition, infected ferrets were able to transmit the virus to na ferrets through aerosol transmission (Yasushi et al. 2009).

Intranasal inoculation of turkeys, chickens, Japanese quail, and domestic ducks produced no clinical signs. Viral replication was only detected in Japanese quail and there was no transmission to other contact birds (Swayne et al. 2009).

3 pH1N1 in Swine

On May 2, 2009, the first confirmed case of pH1N1 in animals was reported to the World Organization for Animal Health (OIE). A small swine herd in Alberta, Canada, displayed typical influenza symptoms after a barn worker had returned from Mexico with an influenza-like illness in early April. A public health investigation concluded that transmission from people to pigs was the likely source of infection (Howden et al. 2009).

Reported symptoms included an acute onset of deep non productive coughing with abdominal effort, occasional sneezing, clear oculonasal discharge, and mild conjunctivitis in about 25 % of the growing pigs. Anorexia, lethargy, pyrexia, and reduced growth were also noted. Mortality increased from 0.19 to 2.04 % in one affected room, and from 0.43 to 0.87 % in a second room. Clinical signs were seen in all the grow completion and nursery barns, while the adult sows remained asymptomatic (Howden et al. 2009).

Gross pathological lesions were consistent with bronchopneumonia due to multiple respiratory pathogens. Secondary bacterial infection with purulent exudate in the airways and abscessation was seen in some animals. Tracheitis, broncho-interstitial pneumonia, bronchiolitis, and alveolitis were observed on histopathology. These lesions were more severe than those reported from experimental infection likely due to concurrent infection with other respiratory pathogens such as *Mycoplasma hyopneumoniae* (MH) and porcine respiratory and reproductive virus (PRRS).

This herd was small, geographically isolated, and not vaccinated. There had not been a reported influenza infection in this herd since 2004, thus this herd could be considered na to H1N1 influenza. The clinical signs and production losses were clinically indistinguishable from other commonly circulating swine influenzas and were no more severe (Brockhoff 2010).

After 3 weeks, the symptoms resolved. PCR test results from nasal swabs showed a decline in apparent herd prevalence from 13.6% on May 11th to 7.9 % on May 25th to 1.5 % on June 1st. No virus was isolated after the initial testing on April 28th. Seroconversion of 70.6 % of sampled pigs occurred within 4 weeks, indicating that pigs in all areas were exposed to the virus in a short period of time. This is consistent with the rapid transmission and recovery seen with classical swine influenza viruses. These data indicated that pH1N1 acted very similar to other swine influenza viruses in pigs (Howden et al. 2009).

A second case of pH1N1 in swine in Canada was identified in June, 2009. pH1N1 infection was confirmed by PCR in two symptomatic farm workers. Very mild influenza symptoms appeared in only the adult pigs between 4 and 7 days after contact with the infected workers (Forgie et al. 2011). At the peak of the outbreak, only 4 % of animals displayed clinical signs consistent with influenza (pyrexia, anorexia, coughing, sneezing). PCR results indicated that infection and viral shedding occurred frequently in asymptomatic animals. There was complete recovery within 2 weeks with no associated mortality or sequelae. Production records suggested that there were negative impacts on subsequent growth and reproductive performance in spite of the mild clinical signs (Keenliside et al. 2010).

In contrast to the first case, only adult swine were symptomatic, although virus was isolated from nursery and growing pigs. Sows in this herd were vaccinated for classical H1N1 and H3N2 viruses. Possibly maternal immunity from vaccination protected the younger pigs. Vaccination, the absence of other respiratory pathogens such as MH and PRRS, and the excellent air quality likely contributed to the milder clinical disease here (Keenliside et al. 2010).

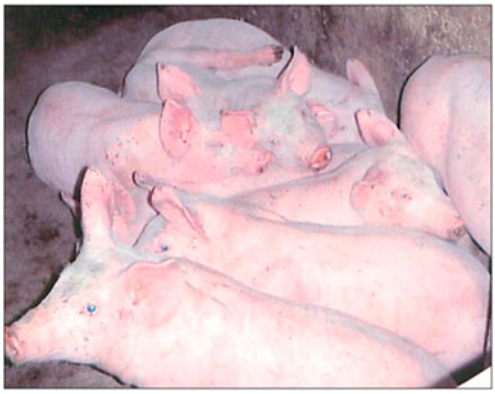
These cases illustrate the variability in symptoms created by influenza viruses in different pig barns and the difficulty in relying on clinical signs alone for surveillance. The symptoms were so mild in the second case that infection could easily escape detection in a less rigorously observed herd (Keenliside et al. 2010).

During the summer of 2009, five swine herds infected with pH1N1 were identified in Manitoba, Canada. Symptoms were mild and similar in duration to infections with classical swine influenza viruses. Shedding of the virus continued for up to 20 days after clinical signs appeared (Pasma and Joseph 2009).

From July to September 2009, pH1N1 infection in swine was subsequently reported to the OIE by Argentina, Australia, Manitoba, Quebec, and Ireland (ProMED-mail 2010a). The clinical picture presented by the other reported cases was consistently one of mild disease indistinguishable from endemic swine influenza viruses. In many cases, there are reports of prior clinical disease or confirmed infection in farm workers, supporting the thesis that humans are the main vector for infection in swine. Serology and virus isolation data from several Korean farms also supported human to swine transmission of pH1N1 (Song et al. 2010).

As of January 2011, 19 countries had notified the OIE of identification of pH1N1 in swine (ProMED-mail 2010a). The virus had been detected though out the world's major swine producing regions within less than one year. Once a country has reported the first case of an emerging disease to the OIE, it is under no obligation to report further cases. Thus, subsequent cases may not be reported and

this record likely significantly underestimates the true number of cases in swine. Some of the reports are from routine surveillance samples rather than clinical cases, indicating the importance of surveillance in detecting cases (ProMED-mail 2010b). 11-week-old pregrower pigs huddling with mild depression and reluctance to rise in the first reported case of pH1N1 in swine (photo courtesy of Egan Brockhoff)



4 pH1N1 in Birds

In August 2009, Chile became the first country to identify pH1N1 in a species other than swine. A commercial turkey breeding farm was confirmed infected, with symptoms of reduced egg production and shell quality. Similar symptoms were seen in three other flocks on the same farm and two other flocks on another breeding farm 50 km away. Salpingitis, peritonitis, and interruption of follicular development were observed on necropsy. Birds recovered and returned to normal lay within 3 weeks. Testing indicated that virus was cleared from the birds after 2–4 weeks. Birds were held under biosafety measures and sent to slaughter after the virus was eliminated. Influenza like illness was reported in staff working on the farms but the source of the virus was not confirmed (Mathieu et al. 2010).

Other outbreaks in turkeys have been reported from Canada (September 2009), USA (November and December 2009), and France (Jan 2010). Clinical signs and outcomes were similar to the Chilean report (ProMED-mail 2010a).

Experimental studies with pH1N1 have failed to produce disease in turkeys using standard inoculation routes such as intransal and intraocular. Intrauterine inoculation has been demonstrated to infect the reproductive tract of the turkey hen resulting in decreased egg production. Turkey breeding hens are handled once a week for intrauterine insemination in order to produce fertile eggs, so this route of exposure is plausible (Pantin-Jackwood et al. 2010).

Pet birds such as psittacines (e.g., parrots, budgerigars) that are in close contact with humans may also be susceptible to pH1N1 infection, but there are no confirmed reports of infection thus far.

5 pH1N1 in Ferrets

Domesticated ferrets (*Mustela putorius furo*) are very susceptible to human influenzas because of similar viral receptors and for this reason are used as models for studying influenza (Matsuoka et al. 2009). The first case of pH1N1 in a pet ferret was reported from Oregon, USA in October 2009 (ProMED-mail 2009a). The owner also reported influenza symptoms prior to the ferret's illness. Three more cases were reported in November from Oregon among a group of nine ferrets. All the animals exhibited mild influenza symptoms (pyrexia, weakness, sneezing, coughing) and recovered uneventfully. The owners were also ill with influenza like illness the week before (ProMED-mail 2009b). Given the mild disease and the popularity of ferrets as pets in North America and Europe, it is likely that more cases of pH1N1 occurred than were reported.

6 pH1N1 in Canines

In December 2009, China reported detecting pH1N1 in samples from two domestic dogs with clinical signs at the Animal Hospital of the China Agricultural University (ProMED-mail 2009c).

Also in December 2009, the USA confirmed pH1N1 in a 13 year old pet dog from New York. Symptoms included lethargy, coughing, anorexia, pyrexia, and pneumonia. Complete recovery occurred after treatment with fluids and antibiotics and supportive care. The dog became ill after its owner was confirmed with pH1N1 indicating probable human to animal transmission. Tests were negative for canine influenza (H3N8) (ProMED-mail 2009d).

7 pH1N1 in Felines

The first reported case of pH1N1 in a felid was November 2009 in an indoor domestic house cat from Iowa, USA. Two of the three family members were ill prior to the cat. (ProMED-mail 2009e). A second cat was identified with pH1N1 in Iowa, USA later the same month. The owner had previously been confirmed to be infected with pH1N1. Both cats recovered. A third case was reported in a house cat from Oregon USA, also in November, however this animal died presumably as a

result from the infection. A fourth case, also from Oregon, also died. Its owner was confirmed infected with pH1N1 previously (ProMED-mail 2009f).

A cheetah (*Acinonyx jubatus*) at a wildlife park in California USA exhibited typical influenza symptoms and was confirmed to be infected with pH1N1 (ProMED-mail Dec 2009a).

8 pH1N1 in Wildlife

Two striped skunks (*Mephitis mephitis*) were confirmed to be infected with pH1N1 virus in Canada in 2009. They were part of a group of eight skunks that died on a commercial mink (*Neovison vison*) farm. Symptoms included purulent nasal exudate, splenomegaly, and severe bronchopneumonia. Nasal discharge was also observed in the mink, suggesting that they may have been infected. Workers on the mink farm did not report any illness. The skunks visited the mink farm daily, making transmission from humans to minks to skunks a possibility (Britton et al. 2011).

A California zoo reported respiratory illness in an American badger (*Taxidea taxus taxus*), a Bornean binturong (*Arctictis binturong penicillatus*) and a black-footed ferret (*Mustela nigripes*). Clinical signs were typical of influenza and included inappetance, lethargy, coughing, nasal discharge, dyspnea. The badger and binturong were euthanized while the ferret survived. PCR detected p H1N1 protein in all three animals. No other pathogens were detected and the animals were otherwise healthy. Pulmonary lesions consisted of interstitial pneumonia with diffuse alveolar damage. All three animals had contact with human caretakers although no influenza like illness was reported in the caretakers (Schrenzel et al. 2011).

A previous serosurvey of 730 wild raccoons (*Procyon lotor*) from seven states in the USA identified antibodies to H1, H3, H4N2, H4N6, and H10N7 influenza subtypes in 2.4 % of the animals. Raccoons, like skunks have receptors for both avian and human influenzas. It is therefore plausible that raccoons could also be infected with pH1N1. Both raccoons and skunks are common mammals that frequently travel between agricultural operations, forests, and urban areas. Thus, wildlife could potentially act as vectors for transmission of influenza viruses to or from poultry and swine operations (Hall et al. 2008).

9 Food Safety

Early in the pandemic, concern was raised over the potential for the zoonotic transmission of the pH1N1 influenza virus via the consumption of pork from infected swine. This concern led to temporary reductions in pork purchases by consumers in various countries, the refusal of some processors to buy pigs from known infected barns and the closure of some international borders to pork from infected countries (Gietz 2010).

Three experimental studies have shown that in swine, the virus is restricted to respiratory organs. No viral RNA was detect by PCR in serum, lung, tonsil, liver, kidney, spleen, inguinal lymph node, colon contents (feces), and skeletal muscle from the *semitendinosus* at days three, five, and seven post infection from 30 pigs experimentally infected either with A/California/04/09 or A/Mexico/4108/2009. In contrast, virus was isolated from the lung tissue of all infected pigs, and viral RNA was detected. (Vincent et al. 2009). Virus was not detected in plasma samples from pigs experimentally infected with virus A/Regensburg/D6/09 (Lange et al. 2009a, b).

A joint statement issued by the FAO, WHO, and OIE declared that influenza viruses are not known to be transmissible to humans through eating pork products. (World Health Organization 2009a, b, c, d).

10 Prevention and Control

The initial cases of pH1N1 infection in swine herds in several countries resulted in government imposed movement controls on the herd. No animals were allowed to leave the premises or go to slaughter until clinical signs had ceased. Mass slaughter was not seen to be an effective or warranted response given the mildness of the disease and the likelihood that humans were the major source of virus (Keenliside 2010). In the case of the first Canadian herd, local slaughter facilities were reluctant to accept clinically healthy animals for slaughter due to the media attention and pressure from international markets for Canadian pork. The owner opted to have his herd destroyed and repopulated (Howden 2009).

In only two countries (Norway and Egypt) was mass slaughter attempted as a method of control. In Norway several confirmed infected herds were slaughtered. As it became apparent that humans were the source of virus for swine, the culling of infected herds as a control measure was abandoned (ProMED-mail 2009h). In Egypt, the mass slaughtering of the country's 300,000 swine reportedly began in April 2009, before there were many confirmed cases in the country. Human cases of pH1N1 appeared in Egypt in June, 2009 and the reason for the cull was amended to be for general improvement of public health (ProMED-mail 2009i).

After the virus was observed to be mild in swine, and evidence showed meat from infected barns to be safe, governments switched to voluntary movement control. Animals could go to slaughter and enter the human food chain once they were asymptomatic. By May 2009 the WHO stated there was no justification for trade restrictions on pigs or pork products for this virus (WHO 2009; Keenliside 2010).

Vaccines for influenza have been available for swine more than two decades. Commercial vaccines typically contain the endemic H1N1 and H3N2 strains, but some have been updated to contain the pH1N1 strain. In regions where the density of swine populations is high and the risk of influenza infection is therefore high, swine are routinely vaccinated for influenza. In regions where the risk is less, or

the cost outweighs the benefit, routine influenza vaccination is not practiced. Other species of livestock and pets are not routinely vaccinated for H1 influenza.

The potential for cross species transmission leading to the mixing of viruses and the development of novel human pandemic strains remains a concern. The Centers for Disease Control and Prevention (CDC) has issued guidelines for workers entering barns where swine are known to be infected. Workers are advised to wear personal protective equipment (PPE) such as masks and gloves and to be vaccinated with seasonal influenza vaccines. Swine farmers are recommended to test suspect barns for confirmation of infection and report the infection. People are advised not to enter pig barns if they have symptoms of influenza in order to prevent human to animal transmission (CDC 2009).

Given that both humans and swine can shed the virus asymptomatically, these precautions may not be completely effective. Ongoing surveillance of swine viruses for changes in genetic makeup has been recommended.

11 Impact of pH1N1 on Livestock Production

The naming of the pH1N1 virus as "swine flu" in the international media led to an immediate drop in the demand for pork in many regions of the world and subsequently price of pork. Estimates of losses to pork producers in North America run into hundreds of millions of dollars. Several countries shut their borders to imports of pork from North America for months afterwards. Communication efforts on behalf of the pork industry helped reassure consumers and markets did recover (Gietz 2010).

Movement control orders create economic loss and animal welfare challenges for livestock producers. Pigs grow rapidly and within 4–6 weeks of the application of a movement control order a pig barn may be critically overcrowded. If animals cannot leave the premises, euthanasia on site will be required. (Bargen and Whiting 2002). Given that pH1N1 is a mild disease that resolves rapidly, and that the economic consequences of reporting could be severe, pig producers can be reluctant to report suspect cases or pursue confirmatory diagnostics. Consequently, the number of reported cases in swine is most likely well below the actual number of cases. Anecdotal reports suggest infection is now widespread in the commercial swine industry across the globe.

Increased surveillance of swine populations for influenza viruses has been suggested as a control measure against the development of future pandemic viruses (WHO). However, in order to be successful, future surveillance and reporting policies must include provisions to protect the livelihoods of farmers and veterinarians in the event of a positive test (Branswell 2010; Keenliside 2010).

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Therapeutics Against Influenza

Elena A. Govorkova and Jonathan A. McCullers

Abstract Despite 75 years of research into prevention and treatment of influenza, the viruses that cause this disease continue to rank as some of the most important pathogens afflicting humans today. Progress in development of therapeutics for influenza has been slow for much of that time, but has accelerated in pace over the last two decades. Two classes of antiviral medications are used in humans at present, but each has limitations in scope and effectiveness of use. New strategies involving these licensed agents, including alternate forms of delivery and combination therapy with other drugs, are currently being explored. In addition, several novel antiviral compounds are in various clinical phases of development. Together with strategies designed to target the virus itself, new approaches to interrupt host–pathogen interactions or modulate detrimental aspects of the immune response have been proposed. Therapy for influenza will likely undergo substantial changes in the decades to come, evolving with our knowledge of pathogenesis as new approaches become viable and are validated clinically.

Contents

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Current Topics in Microbiology and Immunology (2013) 370: 273–300
DOI: 10.1007/82_2011_198

Published Online: 14 January 2012

1 Introduction

Influenza is a contagious respiratory illness caused by influenza viruses. Every year, influenza epidemics cause numerous deaths and millions of hospitalizations. The most frightening effects, however, are seen when new strains of the virus emerge from different species causing worldwide outbreaks of infection. In April 2009 a novel influenza virus of H1N1 subtype emerged from a swine reservoir, causing the first pandemic in more than 40 years (Perez-Padilla et al. 2009). The clinical attack rate was highest in children, and children and young adults of school age were the main vectors of transmission (Nishiura et al. 2009). Surprisingly, however, much of the severe disease from the new pandemic strain also occurred in school-age children and young adults, groups that are typically spared by the most serious outcomes during seasonal influenza (Reichert et al. 2010). Despite the unexpected emergence of a pandemic H1N1 strain, significant concern remains over the potential of highly pathogenic avian influenza viruses of the H5N1 subtype to emerge and achieve similar worldwide spread (Webby and Webster 2003; McCullers 2008). Although transmission of H5N1 influenza viruses from birds to humans is currently inefficient, the capacity to infect humans and cause severe pneumonia with rapid progression to acute respiratory distress syndrome and multi-organ failure (Beigel et al. 2005; bdel-Ghafar et al. 2008) suggests a pandemic from one of these viruses might be much more severe than that from the pandemic 2009 H1N1 strain.

Several antiviral compounds have been developed against influenza virus to interfere with specific events in the replication cycle (McCullers 2005). Among these, two classes of drugs are currently approved as antiviral agents by the food and drug administration (FDA) of the United States. The adamantanes are inhibitors of viral uncoating (amantadine, rimantadine), while the neuraminidase (NA) inhibitors (zanamivir, oseltamivir) interfere with the viral budding process. While these drugs are effective in reducing symptomatology from influenza, increasing evidence of resistance to these conventional antiviral drugs and the narrow time window during which

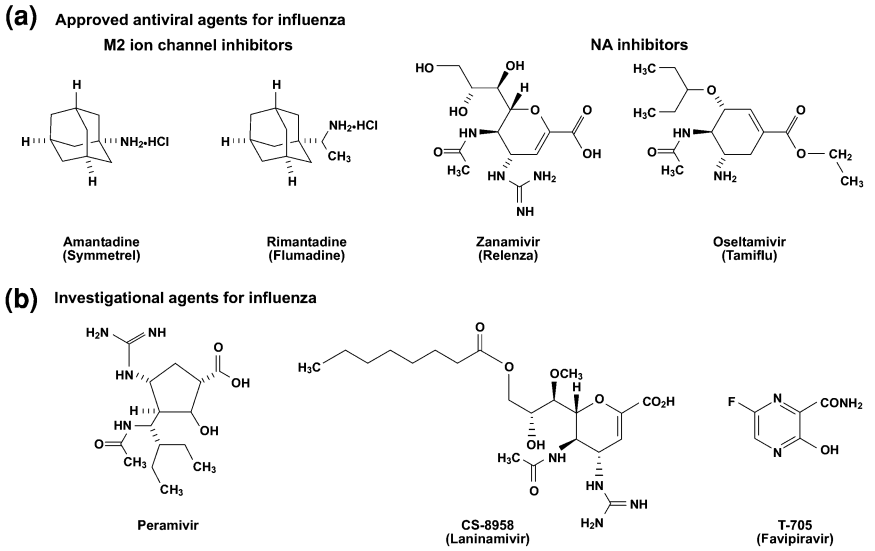

Fig. 1 Structures of antiviral agents active against influenza viruses. **a** Approved antiviral agents for influenza. **b** Investigational agents for influenza

their administration is effective are driving an increased push for novel therapeutic targets, drug combinations, or optimization of the existing antiviral regimens.

2 M2 Ion Channel Inhibitors

2.1 Pharmacokinetics and Clinical Use

The first clinically useful anti-influenza drugs were the adamantane derivatives, amantadine (1-aminoadamantane hydrochloride; trade name Symmetrel) and its methyl derivate rimantadine (α-methyl-1-adamantane-methylamine-hydrochloride; trade name Flumadine) (Fig. 1). The first report of the antiviral activity of amantadine against influenza A viruses was published in 1964 (Davies et al. 1964). Amantadine was initially approved in the United States in 1966, and rimantadine in 1993. Pharmacokinetic studies carried out with healthy immunocompetent adults demonstrated 85–95% oral bioavailability of amantadine and rimantadine and systemic distribution of these drugs with the ability to cross the placenta and the blood–brain barrier; distribution was seen into breast milk, saliva, tears, nasal secretions, and cerebral spinal fluid (Aoki and Sitar 1988). Amantadine is rapidly and almost completely absorbed from gastrointestinal tract with time to peak plasma concentration 2–4 h and a plasma half-life of 17 h (range 10–25 h) (Endo Pharmaceuticals 2007). More than 90% of amantadine is excreted unchanged in urine by glomerular filtration and renal tubular secretion. Rimantadine is also

rapidly absorbed after oral administration, with a time to peak plasma concentration of 5–7 h in healthy adults and a plasma half-life of 25 h (range 13–65 h) (Forest Pharmaceuticals 2010). Unlike amantadine, rimantadine is extensively metabolized in the liver through hydroxylation such that less than 25% is excreted unchanged in the urine. This may account for the lower incidence of central nervous system- related side effects, such as insomnia and difficulty concentrating, that are experienced with rimantadine, compared to amantadine (Dolin et al. 1982). The peak and steady-state concentrations are higher and the half-life of adamantanes is prolonged in patients who are elderly or who have renal impairment (Forest Pharmaceuticals 2010; Endo Pharmaceuticals 2007).

The adamantanes have been shown to be efficacious in the treatment of influenza A virus infection caused by different subtypes (H1N1, H2N2, and H3N2) but are ineffective against influenza B viruses (Table 1) (Wingfield et al. 1969; Doyle et al. 1998; Van Voris et al. 1981; Hayden and Monto 1986). Defervescence, improvement in symptoms, resolution of symptoms, and return to normal activity all occurred about 1 day earlier in treated subjects than in those receiving placebo. Although no studies of sufficient size have been performed to convincingly address whether adamantanes treatment prevents complications of influenza, animal data (McCullers 2004) and a challenge trial in adult volunteers (Doyle et al. 1998) suggest that there is a lack of effect. The adult therapeutic regimen of amantadine or rimantadine is 200 mg/day either as a single dose or divided twice daily for 7 days, and for best therapeutic effect should be administered within 48 h of onset of symptoms (Harper et al. 2009). Efficacy in populations other than healthy adults or when administration is delayed beyond 48 h has not been studied thoroughly. Prophylaxis of healthy adults during influenza outbreaks showed 71–91% efficacy compared to placebo in preventing laboratory-confirmed influenza virus infection in two trials using amantadine (Monto et al. 1979; Dolin et al. 1982), and 85% efficacy using rimantadine in a single trial (Dolin et al. 1982). Prophylactic administration at a dose of 100 mg/dose once daily can be used for up to 6 weeks or until active immunity can be expected from immunization with inactivated influenza A virus vaccine. A recent meta-analysis of published clinical data concluded that the major effects of amantadine and rimantadine treatment were to shorten the duration of fever by about 1 day in treated, infected individuals, and prevent ~60–70% of influenza cases when used as prophylaxis (Jefferson et al. 2010).

2.2 Mechanism of Action

Adamantanes (amantadine and rimantadine) possess two concentration-dependant mechanisms of antiviral action (Pinto et al. 1992). At micromolar concentrations (0.1–5 μM) adamantanes selectively inhibit two different steps in the replication cycle in a strain-specific manner (Appleyard 1977). Prior to membrane fusion, the low pH of the endosome activates the M2 channel to conduct protons across the viral envelope, which results in the acidification of the viral interior. Adamantanes block the ion channel activity of the M2 protein of influenza A virus, and viral

Table 1 Approved antiviral agents for influenza

Characteristics	M2 Inhibitors		NA Inhibitors	
	Amantadine	Rimantadine	Zanamivir	Oseltamivir
FDA approved	1966 Symmetrel	1993 Flumadine	1999 Relenza	1999 Tamiflu
Efficacy	Influenza A virus infection		Influenza A and B virus infection	
Treatment regimen (adults)	100 mg orally bid × 5 days	100 mg orally bid × 5 days	2 inhaled doses (10 mg) bid × 5 days	75 mg orally bid × 5 days
Treatment regimen (children)	5 mg/kg/d orally (max 150 mg/d) in 2 doses × 5 days	5 mg/kg/d orally (max 150 mg/d) in 2 doses × 5 days	>7 years 2 inhalaled doses (10 mg) bid × 5 days	>1 year 12–75 mg orally bid × 5 days
Prophylaxis regimen (adults)	100 mg orally bid	100 mg orally bid	2 inhalaled doses (10 mg) qd × 10 days	75 mg orally qd × 10 days
Prophylaxis regimen (children)	5 mg/kg/d orally (max 150 mg/d) in 2 doses	5 mg/kg/d orally (max 150 mg/d) in 2 doses	>5 years 2 inhalat. (10 mg) qd × 10 days	>1 year 12–75 mg orally qd × 10 days
Adverse effects	Nausea, dizziness, insomnia, vomiting, nervousness, light headedness, impaired concentration, seizures	Nausea, dizziness, insomnia, vomiting, light headedness; less pronounced CNS adverse effects	Bronchospasm	Nasal congestion, nausea, vomiting, discomfort
Mechanism of action	Inhibit viral replication by blocking the ion channel at the stage of virus entry into cells; prevent virus release by altering the conformation of the HA protein		Block the activity of the NA enzyme and interrupt an established infection at a later stage of virus replication by inhibiting the release of virions from infected cells	
Molecular markers of resistance[a]	Mutations in M2 protein at positions: L26F; V27A/T/S/G; A30V/T/S; S31N/G; G34E		Mutations in NA: Q136K (N1and N2 subtypes); E119D/G/A (B virus)	Mutations in NA: H275Y, N295S (N1 subtype); E119V, R292K (N2 subtype); R152K, D198N (B virus)

Note N1 and N2 numbering is used to designated NA mutations in corresponding NA subtypes of influenza A viruses; N2 numbering is used to designated NA mutations for influenza B viruses. [a] Based on sequence analysis of M gene (M2 inhibitors) or NA gene (NA inhibitors). Q136KNA mutation has been reported in MDCK-propagated clinical isolates of seasonal H1N1 and H3N2 viruses with the reduced susceptibility to zanamivir (Hurt et al. 2009; Dapat et al. 2010)

replication is inhibited by the blockade of hydrogen ion flow into the virus particle, principally at the stage of virus entry and uncoating (Wang et al. 1993). Amantadine also acts at a late stage of replication by preventing virus release of certain influenza strains that possess intracellularly cleavable hemagglutinin (HA), in particular the H5 and H7 subtypes. This effect is proposed to result from irreversible conversion of the HA to its low-pH conformation form within the trans-Golgi network in the absence of M2 function (Grambas et al. 1992; Betakova et al. 2005). When cells are incubated in vitro with adamantanes at concentrations >0.1 mM, amantadine and rimantadine are concentrated in lysosomes, and the acid-dependent activation of HA-mediated membrane fusion is inhibited through an increase in the lysosomal pH (Gething et al. 1986; Steinhauer et al. 1996). However, the clinical utility of this second effect is not known as it is not thought to occur at deliverable drug concentrations.

2.3 Resistance

Rapid development of fully pathogenic and transmissible resistant variants after amantadine or rimantadine treatment and their ineffectiveness against influenza B virus infection are the main drawbacks of M2 blockers (Hayden 1996). The markers of resistance to adamantanes are well established and include substitution of one of five amino acids (positions 26, 27, 30, 31, and 34) within the transmembrane domain of M2 protein (Table 1); each change confers resistance to both amantadine and rimantadine (Hay et al. 1986; Belshe et al. 1988; Pinto et al. 1992). Amantadine-resistant influenza A viruses are found among 30–80% of isolates after only a few days of drug therapy in both immunocompetent and immunocompromised patients (Shiraishi et al. 2003).

The incidence of naturally occurring amantadine-resistant variants has increased dramatically since 2003, and these resistant influenza A (H1N1) and A (H3N2) viruses spread widely and reached nearly 100% even in countries without substantial amantadine use (Bright et al. 2006; Centers for Disease Control 2006). However, it is important to note that the percentage of resistant variants varies in different countries and among different influenza A virus subtypes. With the decrease in the use of adamantanes over the last several years driven by these trends in susceptibility, resistance to adamantanes in seasonal influenza A (H1N1) viruses from the United States has returned to relatively low levels, 10.7 and 0.7% for the 2007–2008 and 2008–2009 seasons, respectively (Centers for Disease Control 2010). Conversely, adamantane resistance in South East Asia has remained elevated (33–100%) since 2007 (Barr et al. 2008). Phylogenetically, the M genes of amantadine-sensitive and amantadine-resistant influenza A (H1N1) viruses form two distinct clades: 2B and 2C, respectively (Deyde et al. 2007). Among recent seasonal influenza A (H1N1) viruses, clade 2C viruses carry the S31N mutation in the M2 protein, the most commonly detected amantadine resistance marker, while clade 2B viruses are primarily amantadine sensitive. If amantadine resistance is detected in the primarily amantadine-sensitive clade 2B viruses, it is usually linked with the development of

resistance during amantadine treatment (Hayden 2006). The S31N mutation is also present in current seasonal A (H3N2) viruses. Widespread circulation of amantadine resistance has been prevalent in influenza A (H3N2) viruses during recent seasons (33 and 100% for the 2007–2008 and 2008–2009 seasons, respectively) despite the decrease in usage of the drug (Bright et al. 2005; Barr et al. 2007, 2008). However, amantadine resistance in A (H3N2) viruses was acquired independently and may be related to the collective effects of drug pressure, spontaneous mutations, or reassortments in the viral genome (Deyde et al. 2009; Nelson et al. 2009). For this reason, the United States Centers for Disease Control and Prevention (CDC) has discouraged use of M2 inhibitors until the frequency of this phenotype has subsided (Centers for Disease Control 2006).

3 NA Inhibitors

3.1 Pharmacokinetics and Clinical Use

Development of the NA inhibitors was a significant milestone in antiviral development as this was the first example of synthesis of such a drug based on the crystal structure of a target enzyme (von Itzstein et al. 1996; Kim et al. 1997; Babu et al. 2000). The NA inhibitor zanamivir (4-guanidino-Neu5Ac2en, GG167, trade name Relenza) was designed to be a competitive inhibitor of sialidases (Fig. 1). A second NA inhibitor developed shortly after zanamivir, the prodrug oseltamivir phosphate (oseltamivir) (ethyl[3R,4R,5S]-4-acetamido-5-amino-3-[1-ethylpropoxy]-1-cyclohexene-1-carboxylate, trade name Tamiflu), is rapidly cleaved into the active oseltamivir carboxylate ([*3R,4R,5S*]-4-acetamido-5-amino-3-[1-ethylpropoxy]-1-cyclohexene-1-carboxylic acid) by esterases in the gastrointestinal tract, liver, or blood (Gubareva et al. 2000; McClellan and Perry 2001). The NA inhibitors were approved by FDA for the treatment and prevention of influenza in 1999. The oral bioavailability of zanamivir is <5%, which led to development of a dry powder formulation for inhalation (5 mg zanamivir per 20 mg lactose) (Cass et al. 1999). Systemic absorption was improved by this route, with about 15% total bioavailability, a time to peak plasma concentration of 1–2 h, and a plasma half-life of 3–5 h. More than 90% of absorbed zanamivir is excreted unchanged in the urine (Cass et al. 1999). Oseltamivir carboxylate has oral bioavailability ~80%, peak plasma concentration is achieved 3–4 h after administration, and the plasma half-life is 6–10 h (McClellan and Perry 2001). Oseltamivir is not thought to distribute into the brain (Straumanis et al. 2002), although central nervous system toxicity in juvenile rats with an immature blood-brain barrier has led to caution in the use of this agent in children under 1 year of age (Kimberlin et al. 2010). Oseltamivir carboxylate is eliminated primarily by renal excretion through a combination of glomerular filtration and anionic renal tubular secretion (He et al. 1999). In general, adverse events after oral administration of oseltamivir are considered to be mild and include nausea and vomiting. Inhalation of zanamivir is

generally well tolerated but may cause bronchospasm in some patients with underlying lung disease (Gubareva et al. 2000).

Both oseltamivir and zanamivir are effective in early treatment of influenza A viruses in experimentally infected volunteers (Hayden et al. 1999b; Calfee et al. 1999), and were effective and well tolerated in adults treated for natural influenza infection (Hayden et al. 1997; Treanor et al. 2000; Nicholson et al. 2000). Reduced effectiveness for influenza B viruses as compared to influenza A viruses has been reported for oseltamivir (Kawai et al. 2008), and, in general, only sparse data are available from randomized trials of NA inhibitor effectiveness against influenza B viruses. The therapeutic benefits of NA inhibitors have been reported to include reductions of about 24 h in the time to alleviation of illness, resumption of usual activities, and duration of fever, as well as decreases in illness severity, ancillary medication use, viral titers, and the frequency of antibiotic prescriptions for lower respiratory complications (Hayden et al. 1997; Nicholson et al. 2000; Treanor et al. 2000). Oseltamivir both decreases the incidence of secondary bacterial pneumonia and reduces the severity of complications in an animal model (McCullers 2004). Similar data are not available from a single, well-powered trial in humans, although a meta-analysis of data from multiple trials including unpublished data suggests these results can be extrapolated at least to healthy adults (Kaiser et al. 2003). In children, however, oseltamivir was shown to reduce the occurrence of otitis media by 44% compared to placebo (Whitley et al. 2001). Retrospective reviews of insurance claims databases suggest that NA inhibitors reduce the risk of otitis media, pneumonia, respiratory illnesses other than pneumonia, and hospitalization in both adults and children (Gums et al. 2008; Piedra et al. 2009). Limited data are also available on reduction of risk in adult diabetics, where fewer respiratory illnesses and hospitalizations were noted using this methodology (Orzeck et al. 2007).

Both zanamivir and oseltamivir have been shown to be efficacious in preventing laboratory-confirmed influenza in healthy adults during an outbreak of influenza (Monto et al. 1999; Hayden et al. 1999a) and have demonstrated the ability to interrupt household transmission (Welliver et al. 2001; Hayden et al. 2000, 2004). Zanamivir is approved for the treatment of acute influenza in adults and in children 7 years and older with a recommended dosage of 10 mg twice daily for 5 days by inhalation (Harper et al. 2009). Oseltamivir is indicated for the treatment of acute influenza in patients aged >1 year, and is administered orally to adults at 75 mg twice daily for 5 days starting within 2 days of symptom onset (Table 1). The oseltamivir treatment dosage for children of age 1–12 is based on weight. Early administration of oseltamivir increases the benefit seen in healthy adults relative to treatment at 48 h (Aoki et al. 2003), but no randomized, controlled trials have been conducted studying treatment outside of the first 48 h , so no data are available to examine the effects of late treatment on prevention of complications. Indeed, since persons with chronic illness, who might be more likely to benefit from late treatment as viral control might be established later in such individuals, have typically been excluded from antiviral studies this question is currently unanswered.

Zanamivir is approved for prophylaxis in adults and in children 5 years and older, using a single daily 10 mg dose for 10 days for household prophylaxis and

for 28 days for seasonal prophylaxis (Harper et al. 2009). Oseltamivir is also approved for prophylaxis of influenza at a dosage of 75 mg per day for up to 6 weeks (Harper et al. 2009). Higher doses and longer durations of therapy with oseltamivir (150 mg twice daily for 10 days) have been attempted anecdotally for severe infections with H5N1 subtype viruses or in immunocompromised subjects (Beigel et al. 2005; Le et al. 2005; Memoli et al. 2010), but no data from randomized trials are available to assess the effectiveness of these measures. For seasonal influenza, the usage of high dosages of oseltamivir (up to 450 mg twice daily) have been addressed in a pilot manufacturer-sponsored study, showing dose-linear pharmacokinetics and good tolerability (Dutkowski et al. 2010).

3.2 Mechanism of Action

The NA is second to HA as the most abundant protein on the viral surface, with 50–100 molecules per virion. Although the NA and HA surface glycoproteins of influenza virus evolve and change continuously, conserved residues were identified through all influenza subtypes at the NA active site (Colman 1994). The NA contains 19 residues at the active site that are conserved in all NA subtypes of influenza viruses, including eight catalytic residues (R118, D151, R152, R224, E276, R292, R371, and Y406; N2 numbering) that directly interact with the substrate (sialic acid) and 11 framework residues for functional binding and catalysis (E119, R156, W178, S179, D/N198, I222, E227, H274, E277, N294, and E425) that support the catalytic residues (Colman et al. 1993). The NA inhibitors were designed based on the knowledge of the three-dimensional structure of the NA complex with sialic acid (von Itzstein et al. 1996).

The primary function of the NA enzyme in the life cycle of influenza viruses is to cleave the α-ketosidic bond linking the terminal sialic acid residue from the glycoconjugate, destroying the receptor association with the HA (Gubareva et al. 2000). In this manner the influenza viral NA removes sialic acid residues from the surface of the infected cell and from mucins in the respiratory tract, facilitating the release of newly synthesized virus particles and allowing the virus to spread (Matrosovich et al. 2004). The cleavage of HA receptors by NA is also essential to prevent attachment of new viruses to one another and to glycopeptides present on the cell membrane (Colman 1994).

3.3 Resistance

Two mechanisms of resistance to NA inhibitors have been described. The first is mutations within the NA enzyme catalytic site that disrupt a direct interaction with the drug. The second is mutations in the HA that reduce affinity for its receptor, thus compensating for the effect of the drug on NA activity (Gubareva et al. 2000).

Thus, the molecular determinants of NA inhibitor resistance have been mapped not only to NA but also to HA (Gubareva et al. 2000; McKimm-Breschkin 2000). However, mutations at conserved NA residues are reported to be more clinically relevant (McKimm-Breschkin 2002). NA mutations that confer NA inhibitor resistance reduce sialidase activity and/or stability (Staschke et al. 1995; Tai et al. 1998), but the in vitro replication kinetics of these variants do not always reflect the defective NA enzymatic activity. In some cases the replication efficiency of such mutants may be comparable to that of the wild-type virus (Gubareva et al. 1997) or may be compromised (Ives et al. 2002; Tai et al. 1998) in cell culture. The presence of HA mutations that mask the NA defect and the lack of an optimal cell line may limit characterization of the NA inhibitor-resistant variants in vitro (Matrosovich et al. 2003). Sequence analysis of clinically derived drug-resistant viruses revealed that these NA mutations are NA subtype specific and differ with the NA inhibitor used (Ferraris and Lina 2008). The most frequently observed mutations for NA inhibitor-resistant variants for influenza A viruses of N1 NA subtype are H275Y and N295S (N1 numbering); for influenza A viruses of N2 NA subtype are R292K and E119V (N2 numbering), and for influenza B viruses are R152 and D198 N. Initial studies found that NA inhibitor-resistant influenza viruses were severely compromised in vitro and in animal models (Carr et al. 2002; Ives et al. 2002; Herlocher et al. 2002), and thus led to the idea that resistant viruses were unlikely to have an impact on epidemic and pandemic influenza. However, further studies showed that clinically derived H1N1 virus with H275Y NA mutation (Herlocher et al. 2004) and reverse genetically derived H3N2 virus with E119V NA mutation (Yen et al. 2005) possess similar biological fitness and transmissibility as their drug-sensitive counterparts.

Prior to the 2007–2008 influenza season oseltamivir-resistant variants were found in only a small proportion of patients (approximately 4–8% of children and <1% of adults) after treatment with the NA inhibitor (Stilianakis et al. 2002). However, rigorous detection techniques identified resistant mutants in 9 of 50 (18%) Japanese children during treatment with oseltamivir (Kiso et al. 2004). High level of oseltamivir resistance among influenza H1N1 viruses was reported in many European countries starting in the 2007–2008 influenza season. The emergence and widespread of naturally occurring oseltamivir-resistant variants with H275Y NA amino acid substitution among seasonal H1N1 influenza viruses of A/Solomon Islands/3/06-lineage emphasized that drug-resistant viruses can be highly fit and transmissible in humans (Lackenby et al. 2008; Dharan et al. 2009).

During the 2009 H1N1 influenza pandemic, almost all tested viruses remained susceptible to oseltamivir and zanamivir. Oseltamivir-resistant variants with H275Y NA mutation were isolated from individuals receiving prophylaxis (Baz et al. 2009) and from immunocompromised patients (Centers for Disease Control 2009) under drug selection pressure. Oseltamivir-resistant variants also have been isolated from untreated patients (Leung et al. 2009; Zonis et al. 2010) and from a few community clusters (Le et al. 2010). Two cases of suspected nosocomial transmission between immunocompromised patients have been reported, although it is uncertain whether the mutants came from secondary transmission or arose spontaneously

(Gulland 2009). The reasons for the relative paucity of resistant strains and the lack of widespread transmission are not yet clear. However, experimental evidence suggests that the oseltamivir-resistant H275Y mutant of the pandemic 2009 H1N1 virus retained efficient transmission through direct contact in a ferret model, but respiratory droplet transmission was decreased as compared to an oseltamivir-sensitive virus (Duan et al. 2010). This suggests that transmission efficiency of the mutants may be decreased, limiting spread between humans.

4 Changes in Antiviral Policy During 2009 H1N1 Pandemic

The emergence of a novel pandemic strain in 2009 presented several dilemmas regarding the use of antiviral medications for influenza. First, the two licensed classes of drugs, the M2 ion channel inhibitors and NA inhibitors, were only approved for use with acute, uncomplicated influenza within the first 48 h of illness. The major effects of traditional antiviral therapy are symptom reduction (Hayden et al. 1997; Treanor et al. 2000), and earlier treatment is more likely to have beneficial effects (Aoki et al. 2003). During the pandemic, it became apparent that clinical use extended beyond acute, uncomplicated influenza to include severely ill patients with complex, prolonged infections, with treatment often starting beyond the 48 h window (Memoli et al. 2010; Harter et al. 2010). As discussed above, there are limited data on the use of antiviral drugs in hospitalized patients or on the effectiveness of such compounds at preventing complications of influenza. Treatment of such critically ill patients highlighted a second issue that no approved drugs could be given by the intravenous route, which is required in severely ill persons. And finally, resistance complicated management. The pandemic 2009 H1N1 strain was resistant to the adamantanes, but susceptible to the NA inhibitors. Seasonal H1N1 viruses circulating during the 2008–2009 season showed the opposite pattern, susceptibility to adamantanes but resistance to NA inhibitors. Thus, without the ability to not only diagnose but also serotype viruses in the community, the choice of antiviral for empiric therapy was unclear. Furthermore, resistance to NA inhibitors was noted to develop in select circumstances such as prolonged treatment of immunocompromised patients (Memoli et al. 2010), threatening to make all approved agents useless.

In response to these concerns, public health authorities made a number of changes to antiviral policy during the 2009–2010 influenza season. In April 2009, the Department of Health and Human Services (DHHS) issued an Emergency Use Authorization (EUA) for oseltamivir, allowing it to be used in children under 1 year of age, and in patients who were symptomatic for more than 48 h, and hospitalized or severely ill patients. A similar EUA was issued for zanamivir, allowing its use in hospitalized patients and in those after 48 h of onset of symptoms. In addition, an intravenous formulation of zanamivir was made available by the company that produces it on a compassionate use basis for patients whose medical conditions did not allow to use oral or inhaled drugs (Harter et al. 2010). In November 2009, another

EUA was issued for a third, unlicensed NA inhibitor, peramivir (Birnkrant and Cox 2009). Based on an expedited review at FDA, peramivir was thought likely to be effective for treatment of influenza, and was authorized for intravenous administration in hospitalized patients with pandemic H1N1 infection. Since oseltamivir-resistant viruses are typically also resistant to peramivir (Moscona 2009; Memoli et al. 2010), only zanamivir could be used for pandemic H1N1 strains which developed oseltamivir resistance during treatment. In June 2010, the EUAs for oseltamivir, zanamivir, and peramivir were terminated by DHHS upon expiration of the declared emergency related to the 2009 pandemic.

Since patients will continue to have severe disease and complications from influenza, further research is necessary to justify permanent approval for these indications using either existing or novel antiviral compounds. In particular, research is needed on prevention of complications such as bacterial superinfections, viral pneumonia, and cardiac events. Patient populations at highest risk of developing complications and requiring hospitalization, including asthmatics, persons with heart disease, infants, and the elderly, should be targeted in these trials. Hopefully data from use outside of the normal indications will become available from the experience during the 2009 H1N1 influenza pandemic.

5 Combination Therapy

The segmented genome of influenza viruses, allowing reassortment between viruses, and a high mutation rate based on infidelity of the viral polymerase are factors in the emergence of resistance to any single antiviral drug therapy. Resistance may be less of a problem when combination treatment regimens are employed against an infectious agent. This strategy has already proven effective in the management of human immunodeficiency virus (HIV)-infected individuals, where multiple drug combinations of highly active anti-retroviral therapy (HAART) have revolutionized treatment (Raboud et al. 2002; Kuritzkes and Walker 2007). The combined application of antiviral drugs that target multiple distinct functions of the virus possess different modes of action, pharmacokinetics, tolerance profiles, and resistance patterns and make it a logical therapeutic option. The existence of effective antiviral agents for influenza combination therapy may not only potentiate antiviral activity and result in synergistic or additive effects, but may also enhance clinical outcomes by allowing reductions of the doses of individual drugs. The major benefit of dose reduction in this scenario is a reduction in dose-related drug toxicity and side effects.

A sufficient body of information is now available on the advantages of double and triple drug combinations on influenza virus infection in cell culture and mouse models. Initial studies included evaluation of adamantanes (amantadine and rimantadine) and the synthetic nucleoside ribavirin. Ribavirin, a broad-spectrum antiviral agent, inhibits influenza A and B virus infection in vitro and in animal models (De Clerq 2006; Smee et al. 2006; Sidwell et al. 2005). In MDCK cells, rimantadine combined with ribavirin showed additive and synergistic effects

against the replication of influenza A viruses (Hayden et al. 1984; Stein et al. 1987). Combinations that paired rimantadine with an NA inhibitor (zanamivir, oseltamivir carboxylate, or peramivir) reduced extracellular H1N1 and H3N2 influenza virus yields in MDCK cells more efficiently than any of the drugs alone (Govorkova et al. 2004). Recent studies have shown highly synergistic activity of a triple combination antiviral drug (TCAD) regimen (oseltamivir carboxylate, amantadine, and ribavirin) against both seasonal viruses and the novel H1N1 pandemic strain in vitro (Nguyen et al. 2009, 2010). The synergy of the TCAD regimen was significantly greater than that of any double combination tested, including a combination comprising two NA inhibitors at concentrations achievable in human plasma (Nguyen et al. 2009). Ribavirin is clinically available in many countries because of its therapeutic activity against hepatitis C virus (Hu et al. 2010). However, it has not been officially approved for use against influenza in the United States, and its approved use is limited in the countries where it is licensed due to a relatively small therapeutic index, induction of hemolytic anemia at high doses, high toxicity, and potential teratogenic effects (De Clerq 2006).

Further, preclinical data from animal models have confirmed the benefits of combination chemotherapy for influenza virus infection (Govorkova and Webster 2010). Combinations of the NA inhibitor peramivir and ribavirin significantly increased survival of mice lethally challenged with influenza A/NWS/33 (H1N1) virus (Smee et al. 2002). A synergistic interaction was reported when rimantadine and oseltamivir were given to mice infected with lethal dose of the 1968 H3N2 pandemic strain (Galabov et al. 2006). Oseltamivir-ribavirin combinations were synergistic against an influenza B virus infection in mice (Smee et al. 2006). Importantly, drug combinations were demonstrated to be efficacious against highly pathogenic H5N1 influenza viruses in vivo (Leneva et al. 2000; Ilyushina et al. 2007, 2008). In a mouse model, oseltamivir combined with amantadine or rimantadine was more effective than monotherapy with oseltamivir in preventing the death of mice infected with H5N1 or H9N2 viruses (Leneva et al. 2000). Combinations of amantadine and oseltamivir produced an additive benefit: survival was 30% with oseltamivir alone, 60% with amantadine alone, and 90% with combination treatment as tested against recombinant amantadine-sensitive A/Vietnam/1203/04 (H5N1) influenza virus (Ilyushina et al. 2007). However, combination therapy was no better than oseltamivir alone against the recombinant amantadine-resistant A/Vietnam/1203/04 (H5N1) influenza virus in this study. An oseltamivir and ribavirin combination therapy showed principally additive efficacy against both clade 1 and clade 2 highly pathogenic H5N1 influenza viruses in a mouse model, although the results were dependent on the H5N1 influenza strain. Higher doses were required to protect mice against A/Turkey/15/06 virus than against A/Vietnam/1203/04 virus (Ilyushina et al. 2008).

Until recently, clinical trials that address the benefits of drug combinations have been limited. The major reason for this lack of clinical study is the high level of resistance to the amantadine among seasonal influenza strains, which had limited the available options using approved agents. A prospective, controlled, trial of oral rimantadine and nebulized zanamivir therapy in seriously ill adults hospitalized

with lower respiratory tract manifestations of influenza was conducted during two influenza seasons (January 1998–April 1999) prior to widespread amantadine resistance (Ison et al. 2003). Patients treated with combinations of the drugs demonstrated a trend toward fewer days of virus shedding and were less likely to have a severe cough. Moreover, no resistant variants were found in the group receiving combination therapy, while 2 of 11 patients in the rimantadine monotherapy group had resistant virus (Ison et al. 2003). In a randomized, crossover study ($n = 17$) it was shown that pharmacokinetics of amantadine (100 mg orally twice daily) were not affected by co-administration of oseltamivir (75 mg orally twice daily), and there was no evidence for an increase in frequency or severity of adverse events when amantadine and oseltamivir were used in combination (Morrison et al. 2007). Given the paucity of clinical data, it is important to now initiate clinical trials specifically designed to evaluate issues regarding combination chemotherapy for influenza. The planning of such studies should include clinical and virological evaluations with determination of influenza virus loads in the patient, the molecular and biological characterization of viruses for resistance and fitness, and pharmacokinetic data to evaluate safety and toxicity.

6 Investigational Agents for Influenza

6.1 Parenteral NA Inhibitors

In addition to further study of existing antivirals, there is an intense need for new antiviral compounds (Hayden 2009). No new influenza antiviral drugs have been approved since 1999 in the United States and none currently have an indication for treatment of severe disease. New formulations of conventional anti-influenza drugs and novel antiviral agents that target either viral proteins or host defense mechanisms are currently at various stages of development (Table 2). Parenteral administration of the NA inhibitors zanamivir (intravenous, IV) and peramivir (IV and intramuscular) is being evaluated in preclinical studies and clinical trials for the treatment of seasonal influenza A infection and were selectively used during the recent 2009 H1N1 pandemic as described above (Harter et al. 2010; Birnkrant and Cox 2009; Memoli et al. 2010). In Japan, parenteral peramivir was licensed under the trade name Rapiacta® in 2010 (Clinical Trials.gov 2010).

6.2 Long-Acting NA Inhibitor

The long-acting inhaled NA inhibitor laninamivir (R-125489 = laninamivir and CS-8958 = laninamivir octanoate or the laninamivir prodrug) is a novel, promising drug for the control of influenza (Fig. 1) (Honda et al. 2009; Koyama et al. 2009).

Table 2 Investigational agents for influenza in clinical development

Agent	Target	Route of administration	Stage of development	Company
Virus-targeted approach				
Relenza (Zanamivir)	NA inhibitor	Parenteral (IV)	Phase 2/3 clinical trials ongoing	GlaxoSmithKline, UK
Tamiflu (Oseltamivir)	NA inhibitor	Parenteral (IV)	Phase 3 trial ongoing	Hoffmann-La Roche, Switzerland
Peramivir	NA inhibitor	Parenteral (IM and IV)	Licensed in Japan as Rapiacta® in January, 2010; Phase 2/3 clinical trials in US ongoing	BioCryst Pharmaceuticals, USA
Laninamivir (CS-8958)	NA inhibitor, long-acting	Inhalation	Phase 3 clinical trial completed	Biota, Australia in partnership with Daiichi-Sankyo, Japan
Favipiravir (T-705)	Polymerase inhibitor	Oral	Phase 2 clinical trial in Japan ongoing	Toyama Chemical, Japan
Triple combination therapy	Combination chemotherapy	Oral	Phase 1–2 clinical trials ongoing	Adamas Pharmaceuticals, Inc., USA
Hyper-immune serum	HA neutralization	Parenteral (IV)	Phase 1–2 clinical trials ongoing	Various
Host-targeted approach				
Fludase®, (DAS181)	Influenza virus receptor inactivator	Inhalation	Phase 1 studies ongoing	NexBio, Inc., USA
Corticosteroids	Anti-inflammatory	Parenteral	Phase 3 study (suspended)	University of Versailles, France
Rosuvastatin	Cholesterol biosynthesis pathway inhibitor	Oral	Phase 3 study ongoing	Vanderbilt University, USA

Note Data from www.clinicaltrials.gov (Clinical Trials.gov 2010). Triple combination therapy is conducted with Symmetrel, Tamiflu and Ribavirin

Laninamivir is a multimeric zanamivir compound that potently inhibited the NA activities of various influenza A and B viruses, including subtypes N1 to N9 and oseltamivir-resistant viruses (Yamashita et al. 2009), as well as the pandemic 2009 H1N1 virus (Itoh et al. 2009). An attractive feature of this compound is the prolonged retention in the lungs which allows once weekly administration and has shown efficacy superior to that of zanamivir and oseltamivir in mouse models of infection with influenza viruses, including seasonal, pandemic 2009 H1N1, and highly pathogenic H5N1 viruses (Koyama et al. 2009; Kubo et al. 2010; Kiso et al. 2010a). Phase 1 clinical trials have been completed in Japan, and no adverse events related to laninamivir octanoate were observed. The drug is slowly eliminated from the body, lasting up to 144 h after administration with a half-life of about 3 days, suggesting that a single inhalation of laninamivir octanoate can act as a long-acting NA inhibitor in humans (Ishizuka et al. 2010). A double-blind, randomized controlled trial demonstrated that the drug was effective and well tolerated in children with seasonal oseltamivir-resistant influenza A (H1N1) virus infection and effective for treatment of disease caused by oseltamivir-resistant influenza viruses (Sugaya and Ohashi 2010).

6.3 Polymerase Inhibitor

Another promising anti-influenza agent which is at advanced stages of development is a substituted pyrazine compound, T-705 (Fig. 1, 6-fluoro-3-hydroxy-2-pyrazinecarboxamide, favipiravir) (Furuta et al. 2002). T-705 inhibits an early to middle stage of viral replication but not the adsorption or release stage. T-705 is converted to the ribofuranosyltriphosphate derivative by host enzymes, and this metabolite selectively inhibits the influenza viral RNA-dependent RNA polymerase in a dose-dependent manner. Interestingly, this compound did not inhibit host DNA and RNA synthesis and only weekly inhibits inosine 5′-monophosphate dehydrogenase (IMPDH) activity (Furuta et al. 2005). T-705 showed a more favorable therapeutic index than did ribavirin in preclinical tests of toxicity in mammalian cells (Furuta et al. 2005). The potent antiviral activity of T-705 in vitro was demonstrated against seasonal influenza A (H1N1, H2N2, and H3N2), B and C viruses (Furuta et al. 2002), influenza A (H5N1) viruses (Sidwell et al. 2007; Kiso et al. 2010b), as well as an oseltamivir-resistant virus (Furuta et al. 2002; Sleeman et al. 2010). Oral treatment with T-705 at the dose of 30 mg/kg/day or more prevented death, inhibited lung consolidation, and reduced lung virus titers in a BALB/c mouse model under lethal challenge with H5N1 and H3N2 subtype viruses (Furuta et al. 2002; Sidwell et al. 2007; Kiso et al. 2010b). In a comparative experiment with oseltamivir, using mice infected with a high challenge dose of influenza A/PR/8/34 (H1N1) virus, T-705 completely prevented death, and the survival rate was significantly higher than in oseltamivir-treated animals (Takahashi et al. 2003). The results of studies of delayed initiation of treatment using influenza A and B viruses showed a marked reduction in mortality even

when treatment with T-705 was initiated from 60 to 96 h post virus inoculation. The benefits of using oseltamivir and T-705 in combination to treat H1N1, H3N2, and H5N1 influenza virus infection were recently demonstrated in a mouse model (Smee et al. 2010). Initial unpublished data on human pharmacology are encouraging with regard to oral absorption and tolerability, and Phase 2 efficacy studies of favipiravir have been conducted in Japan (Furuta et al. 2009).

6.4 HA Inhibitor

Another potential anti-influenza agent is Cyanovirin-N (CV-N), a carbohydrate-binding protein that inhibits viral entry into cells by specifically binding to high mannose oligosaccharides on the surface glycoproteins of enveloped viruses (O'Keefe et al. 2003). CV-N is a 101 amino acid protein derived from the cyanobacterium *Nostoc ellipsosporum* and was originally discovered as an inhibitor of HIV, but was later found to inhibit other enveloped viruses such as influenza and Ebola (Boyd et al. 1997; O'Keefe et al. 2003; Barrientos et al. 2003). CV-N showed antiviral activity against a range of influenza A and B viruses in vitro and in mice and ferrets (Smee et al. 2007, 2008). However, the efficacy was strain-specific and depends on the composition of glycosylation sites on the HA. Loss of these glycosylation sites due to mutation of the HA leads to decreases in CV-N binding and antiviral activity (O'Keefe et al. 2003; Smee et al. 2007). A high mannose oligosaccharide at a conserved residue N94 (H3 numbering, corresponds to position 87 in H1 subtype) of the HA1 subunit of HA is the primary target of CV-N, and substitutions at this position by itself confer CV-N resistance. Mutation(s) that affects the receptor binding site for the HA1 may also reduce efficacy of CV-N against influenza viruses (Smee et al. 2007). Clinical studies in humans have not been reported.

6.5 Sialic Acid Receptor Inhibitor

Targeting the host cell components required for viral infection is a novel antiviral approach which can theoretically lead to lack or low rates of emergence of drug-resistant variants. Sialic acid (SA)-containing receptors on the surface of susceptible cells are required for infection by influenza viruses. The interaction between HA glycoprotein of influenza virus and SA receptors is essential for initial stages of virus replication, suggesting that targeting this interaction as an therapeutic approach would have some promise. DAS181 (Fludase®) is a recombinant fusion protein containing a sialidase catalytic domain and a respiratory epithelium-anchoring domain [amphiregulin (AR) tag], which can be mass produced in *Escherichia coli* (Malakhov et al. 2006). The sialidase activity of DAS181 can cleave SAα2,6- and SAα2,3-linked cellular receptors, which are preferentially

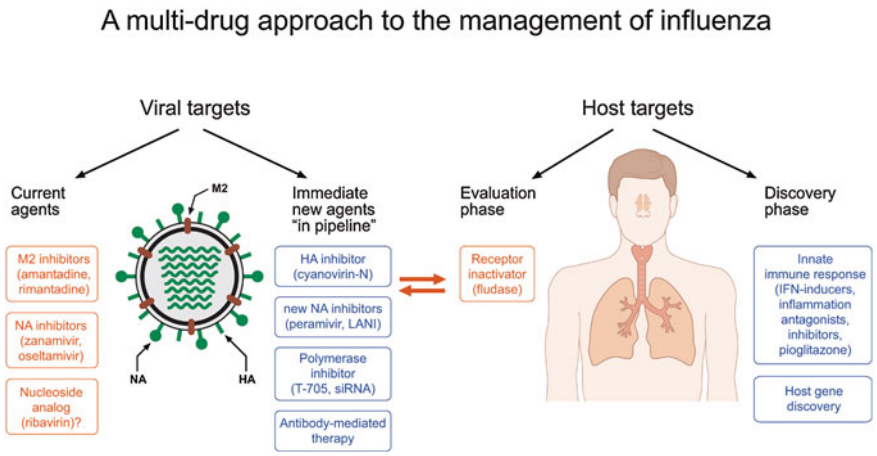

Fig. 2 A multidrug approach to the management of influenza. HA, hemagglutinin; IFN, interferon; LANI, long-acting neuraminidase inhibitor; NA, neuraminidase; siRNA, small interfering RNA. Reprinted from (White et al. 2009) under the terms of the Creative Commons Attributions License

recognized by human and avian influenza strains, respectively. Because it is host-directed toward the SA acid receptors on airway epithelium, it can also prevent the binding of other respiratory viruses that also utilize these receptors (e.g. parainfluenza) (Moscona et al. 2010). DAS181 potently inhibits infection by seasonal influenza A and B viruses, pandemic 2009 H1N1 viruses, NA inhibitor-resistant influenza viruses, as well as the potentially pandemic H5N1 influenza viruses in MDCK cells, mice, and ferrets (Belser et al. 2007; Triana-Baltzer et al. 2009a, b). In vitro removal of receptors by DAS181 leads to a prolonged antiviral effect, although it is not clear whether this effect will translate into a less-frequent dosing regimen in the clinic. Long-term DAS181 exposure to numerous cell lines and human primary cells does not cause cytotoxicity. The resistance potential of this compound requires further investigations. Preliminary data indicated that DAS181-resistant variants could be generated and mild resistance was developed in two out of six strains tested following up to 30 passages in MDCK cells. DAS181-resistant viruses exhibited an attenuated phenotype in vitro and in mice, and still could be inhibited by the higher concentrations of compound (Moss et al. 2010). Phase 1 clinical studies of DAS181 have been completed, but data on safety are not yet publically available.

6.6 Other Candidates

Advances in understanding the mechanisms of influenza virus replication have revealed a number of potential drug targets (Fig. 2). Small interfering RNAs (siRNAs) can be designed to target viral RNA without engaging host RNA, and

therefore can be highly specific, highly effective, have low toxicity, and can be easy to make and formulate (Alvarez et al. 2009). Clinical, proof-of-concept has been shown for an RNA-interference agent targeted against respiratory syncytial virus (DeVincenzo et al. 2010). To this point, only pre-clinical data using RNA inhibitor based therapies are available for influenza (Kumar et al. 2010).

Antibody therapies are another strategy that has been proposed for treatment or prevention of influenza. This includes intravenous immune globulin (IVIG) preparations, which are used clinically for a variety of purposes, hyperimmune sera from recovered or vaccinated individuals, and specific monoclonal antibody therapies (Bearman et al. 2010; Luke et al. 2010; Martinez et al. 2009). Mouse model data using all three approaches suggest efficacy for primary influenza infections (Marinescu et al. 2009; Ramisse et al. 1998; Krause et al. 2010; Kashyap et al. 2010). Limited clinical data in humans support this approach conceptually, primarily from uncontrolled studies of treatment of pandemic influenza or H5N1 virus-infected patients (Luke et al. 2006; Kong and Zhou 2006; Zhou et al. 2007).

The strategy of immunomodulation to broadly reduce the inflammatory response during severe influenza virus infections (Fig. 2) has also been proposed (Fedson 2009), but is currently not supported by clinical data in humans. Systemic steroids were frequently used as a clinical therapeutic during the 2009 H1N1 pandemic (Falagas et al. 2010). In some published studies more than 50% of severely ill patients were treated with corticosteroids (Kumar et al. 2009). However, no clinical benefit of steroids has thus far been shown for ARDS or specifically for influenza (Steinberg et al. 2006; Napolitano et al. 2010). Alternative candidates targeting specific anti-inflammatory pathways have also been put forward (Fedson 2008), including drugs such as statins, which inhibit a cholesterol biosynthesis pathway enzyme (Liu et al. 2009), agonists of peroxisome proliferator-activated receptors including fibrates and thiozolidinediones (Budd et al. 2007; Aldridge, Jr. et al. 2009), cyclooxygenase pathway inhibitors (Zheng et al. 2008), and antioxidants such as N-acetyl-L-cysteine (Geiler et al. 2010). Each has shown some efficacy in mouse models (Budd et al. 2007; Zheng et al. 2008; Aldridge, Jr. et al. 2009; Geiler et al. 2010). Out of this group of candidates, statins are the only agents that have been studied in humans thus far. To this point, however, cohort studies of persons prescribed statins for their cholesterol-lowering properties have shown no obvious clinical benefit against influenza morbidity (Kwong et al. 2009; Fleming et al. 2010). Further, clinical investigation of these agents and their potential to act in combination with traditional antiviral agents needs to be explored.

7 Conclusions

Several issues have limited the effectiveness of the currently available antiviral drugs against influenza. First, unlike antibiotics, which can eliminate or greatly reduce pathogen burden, existing influenza antiviral drugs serve only to halt

progression of disease by preventing new host cells from being infected. If this intervention is administered early enough in the clinical course, it may alter the tempo of infection, allowing normal immune clearance mechanisms to gain the upper hand. Thus, the major effects of treatment are symptom reduction and a more rapid recovery, not immediate clinical cure. Second, the currently licensed antivirals are all oral medications and, until the recent 2009 pandemic, were authorized only for use in mild to moderate influenza. Critically ill patients with either H5N1 or pandemic 2009 H1N1 infections have been difficult to treat until the recent availability of intravenous peramivir and zanamivir. Third, resistance has been a clinically significant issue for the adamantanes for years, limiting their utility. To combat these issues, more research on existing antivirals and further investigation of novel compounds and strategies are needed. Combination therapy has been explored both with existing, licensed, antivirals as well as with agents not currently approved for use against influenza. Novel agents targeting important viral proteins or host-pathogen interactions are at various stages of development. And finally, novel immunomodulatory strategies targeting the virus-mediated effects or host responses are in development and clinical testing. The future of influenza control likely involves improved means to prevent infection, coupled with combined strategies to both slow the virus as well as mitigate the immunopathologic consequences of infection when it occurs. A coalescence of divergent paths of research to meet these goals is needed if the urgent public health threat of seasonal and pandemic influenza is to be met.

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DOI: 10.1007/978-3-642-36871-4