

Bovine Viral Diarrhea Virus

Diagnosis, Management, and Control

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Edited by Sagar M. Goyal and Julia F. Ridpath



*To our families: Krishna, Vipin, Kavitha, Dinesh, Sarina,
Harold, Lance, Reid, and Grant.*

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Preface

The disease caused by bovine viral diarrhea virus (BVDV) was first described in 1946, along with virus isolation from sick cattle. In the intervening 60 years, many important advances have been made in understanding this virus and the disease it produces. These developments include the recognition of BVDV biotypes and genotypes; development of monoclonal antibodies (Mabs) to study strain variation; defining the mechanism of development of persistent infections, mucosal disease, and immune tolerance; BVDV-induced immunosuppression; the discovery of problems caused by the presence of BVDV and anti-BVDV antibodies in fetal bovine serum used in the production of cell cultures; sequencing of viral genome; and the development of rapid methods for virus detection. These achievements have culminated in the development of control and eradication programs in several Scandinavian countries with some successes. Many other

countries, including the U.S., are contemplating eradication and/or control programs.

In spite of these developments and several symposia dedicated to discussing the pathogenesis, transmission, diagnosis, and molecular virology of the virus, BVDV and BVDV-induced diseases are not completely understood, as exemplified by the appearance of severe hemorrhagic disease caused by BVDV 2 in the early 1990s in Canada and then in other countries. It is with this in mind that we have attempted to collate information on the current state of knowledge on the diagnosis, management, and control of the multifaceted diseases caused by BVD viruses. An internationally renowned team of experts has been assembled to contribute chapters on various aspects of this problem.

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1

Introduction and History

Dirk Deregt

In 1996, Cornell University held an international symposium at the College of Veterinary Medicine. This event was a celebration of 50 years of bovine viral diarrhea virus (BVDV) research marked from the time of the first publication on BVDV, “An apparently new transmissible disease of cattle,” by Cornell University researchers (Olafson et al., 1946). Presenting at the meeting were two Cornell pioneers: Dr. Francis Fox, one of the coauthors of the original publication, and Dr. James Gillespie who, with his colleagues, had propagated the first isolates of BVDV in tissue culture. Also presenting at this meeting was another BVDV pioneer, Dr. Bernd Liess of the Hannover Veterinary School, who recounted his early experiences and interactions with Cornell University researchers. Their historical perspectives on the discovery of the disease, the virus, and the scientific contributions of Cornell University are recorded (Fox, 1996; Gillespie, 1996; Liess, 1996). These personal accounts are highly recommended reading. The meeting was a blend of “old” and “new.” Classical (type 1) BVDV was now 50 years old, but what dominated the discussion was the newly discovered genotype of BVDV (type 2 BVDV) and the disease it causes.

From its emergence as a new virus in 1946 to the present day, BVDV has proven to be multifaceted in the disease it produces and is arguably the most complicated bovine virus in its pathogenesis. During the past half-century and more, a multitude of advances have been made in BVDV research and diagnostics. These advances have led to the development of numerous diagnostic tests and the design of successful management and control strategies for BVDV. The discovery of persistent infection and how this state is produced in cattle was key to both our understanding of how BVDV maintains itself and to developing rational control and eradication strategies.

Vaccines were first mass produced in the 1960s to deal effectively with the acute disease but were also found to occasionally precipitate a severe manifestation of BVDV called *mucosal disease (MD)*. The pathogenesis of spontaneous and vaccine-induced mucosal disease in persistently infected cattle came to be understood by important discoveries in the 1960s and 1980s. The decade of the 1980s also saw the first complete sequencing of a BVDV genome and the production of monoclonal antibodies to the virus. These developments led eventually to new nucleic acid- and monoclonal antibody-based diagnostic methods.

In the 1990s, virulent strains of a new BVDV genotype, type 2 BVDV, devastated many North American herds and presented yet another challenge for researchers, veterinarians, and vaccine companies. At the same time, several European countries embarked on eradication programs for BVDV without vaccination. These programs have been highly successful to date. Thus, in the beginning of the new millennium, countries and regions with endemic BVDV will need to consider what type of control programs, if any, they will embark on and what the costs and risks are. In the absence of national or regional programs, veterinarians and cattle producers need to know how to apply proper management and control strategies for individual herds. The objective of this book is to provide detailed information on our current knowledge of the virus and the diseases it causes, the diagnostic methods available, and management systems.

In writing this chapter on the history of BVDV, the author has divided the historical developments into specific time periods. However, to avoid too much fragmentation of thought, some related developments, that either overlap the time periods (earlier or later) or may be too brief to mention individually in their time period, may be brought together in a

single context in the period in which the most significant findings or a majority of the research took place. These events are referenced with their date of publication and, thus, should not be confusing to the reader. In addition to providing a history and introduction to the virus and disease, another objective in writing this chapter is to provide an introduction (in historical context) of subjects that are further developed in subsequent chapters.

THE EARLY YEARS, 1946–1969

THE VIRUS AND THE DISEASE

The new transmissible disease in cattle, described by Cornell University researchers Olafson, MacCallum, and Fox (1946), was characterized by leukopenia, high fever, depression, diarrhea and dehydration, anorexia, salivation, nasal discharge, gastrointestinal erosions, and hemorrhages in various tissues. Initially, the disease was observed in Ithaca, New York, in a “one-cow herd” by Dr. Francis Fox, who initially considered the disease to be classical winter dysentery in his recounting of the event (Fox, 1996). Subsequently, outbreaks of the disease occurred in other herds in the area. In five initial herds, the morbidity ranged from 33–88% and mortality was 4–8%. In addition to other signs, milk production was diminished and fetal abortions occurred 10 days to 3 months following infection. Some of the cows in one herd developed pneumonia. The severe leukopenia seen in clinically affected animals was considered to be indicative of a viral etiology. The attempted treatment involved blood transfusions, but often the apparently healthy donor animals of the herd had a more severe leukopenia than the clinically affected animals (Fox, 1996). This indicated that the donors were infected and that BVDV infections could also be subclinical in nature.

The lesions of BVD resembled those of rinderpest, an exotic disease for the U.S. However, the disease observed by Olafson et al. (1946) did not behave as rinderpest. Since the U.S. had a susceptible cattle population, rinderpest would have presented a much more devastating clinical picture with high transmission and mortality rates. However, it was thought that a mild form of rinderpest could have been responsible for the lesions observed and, thus, it was important to rule out this disease. Subsequently, Walker and Olafson (1947) demonstrated experimentally that sera from cattle that recovered from BVD did not neutralize rinderpest virus and that these recovered cattle did not show resistance to infection with the rinderpest virus.

After the report of acute BVD in New York, a similar but more severe disease in cattle was reported in Canada by Childs (1946). This report was considered by Pritchard (1963) in his review of those early times as probably the first description of mucosal disease in cattle. Mucosal disease was characterized by fever, anorexia, depression, profuse salivation, nasal discharge; gastrointestinal hemorrhages, erosions, and ulcers; and severe diarrhea with watery feces that were sometimes mixed with blood. Lesions of the gastrointestinal tract were quantitatively much more severe in MD than those observed in BVD. Furthermore, MD usually affected only a few animals in a herd but had a very high case fatality rate. In 1953, Ramsey and Chivers (1953) reported on MD in the U.S. and gave the disease its name. These authors noted that MD had some of the characteristics of BVD but was apparently not transmissible experimentally, and only fever was observed in cattle inoculated with the agent. Thus, based on the differences in lesions of the gastrointestinal tract, the low morbidity and high case fatality rates, and the nontransmissibility of MD, it was thought to be a disease distinct from BVD.

It was discovered in 1957 that the viral agent isolated in acute BVD did not cause cytopathology *in vitro*, meaning that infected cells in culture appeared normal (Lee and Gillespie, 1957). In the same year, a cytopathic virus had been isolated from MD (which was thought at the time to be caused by a different virus than the one causing BVD) (Underdahl et al., 1957). After the isolation of two noncytopathic viruses from BVD cases, including the reference NY-1 strain (Lee and Gillespie, 1957), Gillespie et al. (1960) reported the first isolation of a cytopathic strain of BVDV, designated Oregon C24V. The discovery of cytopathic strains allowed the development of serum neutralization and plaque neutralization assays. Cytopathic strains could be more easily studied in tissue culture than noncytopathic strains, and their neutralization with antiserum allowed characterization of the antigenic relatedness of viruses from cases of BVD and MD. Studies employing virus neutralization (Gillespie et al., 1961; Kniazeff et al., 1961; Thomson and Savan, 1963) determined that the viral agents isolated from BVD and MD in North America and Europe were, indeed, the same and that BVD and MD were actually different disease manifestations caused by the same agent. Thus, several years later, the disease became officially known as bovine viral diarrhea-mucosal disease (BVD-MD) (Kennedy et al., 1968).

In an early review of BVD-MD, Pritchard (1963)

described BVD as a separate syndrome from MD and stated that BVD occurs in three forms: severe acute BVD, mild acute BVD, and chronic BVD (now recognized as a form of MD). Severe acute BVD had been prominent a decade earlier, but by 1963, a very mild clinical form of BVD was most commonly observed. Pritchard (1963) noted that one of the common early clinical signs of the severe acute form of BVD was a harsh, dry cough in many of the animals. In some herds, lameness attributed to laminitis was also prominent. In the chronic form, affected cattle failed to grow at a normal rate or lost weight. Many of these animals became emaciated and developed continuous or intermittent diarrhea.

Although BVD could be reproduced experimentally, MD could not. Only fever or a milder form of BVD could be produced with the virus obtained from cases of MD (Pritchard, 1963). An early study of field cases of BVD and MD indicated that there was an immune component to MD (Thomson and Savan, 1963). In seven cases, the cattle that became diseased and died were serologically negative to the virus. One animal, which was clinically ill for 2 months, remained serologically negative immediately prior to death when viable virus was still isolated from its blood. Based on these observations, Thomson and Savan (1963) suggested that cattle that did not recover from their illness may have been incapable of producing an immune response.

By the end of the 1960s, it had become evident that cattle with MD had persistent viremia and often failed to produce neutralizing antibodies to the virus (Malmquist, 1968). It was also observed that fetal bovine serum frequently contained BVDV (Malmquist 1968). This finding and the finding of BVDV infections in newborn and 1-day-old calves were indicative of intrauterine infections (Bürki and Germann, 1964; Romváry, 1965; Malmquist, 1968). Thus, one mechanism proposed to explain persistent infection, and the failure of cattle with MD to produce antibodies to BVDV was the development of immune tolerance in these cattle during intrauterine infection (Malmquist, 1968). Exposure to BVDV before the age of immune competence could lead to failure of the fetus to recognize the virus as foreign and, thus, a failure to produce antibodies to the virus. This would result in the eventual birth of calves infected with BVDV but without the capability to clear the infection. An alternative mechanism proposed by Malmquist (1968) for the lack of neutralizing antibodies in cattle with MD was the destruction of immunologically competent cells by the

virus. It was noted at the time that, if the operating mechanism was immune tolerance, the lack of immune response would be expected to be BVDV-specific, whereas if immune cell destruction was operating, there would also be a depression of the immune response to unrelated antigens (Segre, 1968). The hypothesis of immune tolerance was eventually proven in the 1980s, a decade in which the precipitating event in MD was also identified and in which MD was experimentally reproduced.

Fetal abortions were associated with BVDV infection from the time of the first outbreaks of BVD (Olafson et al., 1946). Evidence of a causative role in abortion included the isolation of noncytopathic BVDV from aborted fetuses and the occurrence of abortions following experimental exposure and after natural outbreaks (Olafson et al., 1946; Baker et al., 1954; Gillespie et al., 1967). Cerebellar hypoplasia and ocular defects (cataracts, retinal degeneration, and optic neuritis) in newborn calves were also observed after BVDV infection of pregnant cows. These BVDV-induced congenital defects were first reported by Ward et al. (1969). For the dams of three calves with cerebellar hypoplasia, Kahrs et al. (1970) reported that infection occurred at an estimated 134–183 days of gestation. Experimentally, cerebellar and ocular defects were produced in calves by inoculation of their dams with a noncytopathic BVDV at 79–150 days of gestation (Scott et al., 1973).

Early in the 1960s, it became established that BVDV was antigenically related to hog cholera virus, now more commonly known as *classical swine fever virus (CSFV)* (Darbyshire, 1962). Soon after, live BVDV vaccines were proposed for immunization of swine against CSFV in the U.S., but this was later abandoned in 1969 when the Department of Agriculture issued a notice against their use for this purpose (Fernelius et al., 1973). Swine had originally been thought to be dead-end hosts for BVDV, making the virus attractive for immunization of swine. However, this was proven to be false when BVDV was isolated from naturally infected swine (Fernelius et al., 1973). Later, serological evidence indicated that the agent causing border disease in sheep was also related to BVDV and CSFV (Plant et al., 1973).

DIAGNOSIS AND CONTROL

The serum neutralization test developed after the discovery of cytopathic strains of BVDV became an important diagnostic assay and is still widely used today. Early studies in the 1960s using this test proved that BVDV was present worldwide at a high

seroprevalence (usually about 60%) in most adult cattle populations and that the majority of BVDV infections were subclinical in nature.

During the 1960s, the use of cell lines such as Madin-Darby bovine kidney (MDBK) cells simplified the study of the virus and its diagnosis (Marcus and Moll, 1968). Also of importance was the development of the fluorescent antibody technique (FAT) for the detection of BVDV in inoculated cell cultures (Fernelius, 1964). Prior to the development of FAT, identification and titration of noncytopathic BVDV was difficult, often involving calf inoculation, an interference test in which the cytopathic effect of cytopathic BVDV was inhibited by noncytopathic virus (Gillespie et al., 1962; Diderholm and Dinter, 1966), or agar gel-diffusion precipitin tests. With FAT, the detection of noncytopathic BVDV from biological specimens was made much easier, although the interference test was still used experimentally as late as 1985 (McClurkin et al., 1985).

Soon after the discovery of the cytopathic BVDV strain, Oregon C24V, Coggins et al. (1961) reported on its attenuation by serial passages in primary bovine kidney tissue culture. This led to its mass production as a modified-live vaccine for BVD in 1964 (Peter et al., 1967). However, soon after its use, reports began to appear of a few sick animals in some herds following immunization. It became apparent that the vaccine produced an MD-like disease in a small number of animals in a few herds (Fuller, 1965; Peter et al., 1967; McKercher et al., 1968). The animals that became sick with MD-like symptoms usually died. The discussion of possible causes of postvaccinal MD (pvMD), as it became known, included insufficient attenuation of the Oregon C24V strain and contamination of the vaccine with a virulent BVDV field strain. Further, since pvMD was first observed after the introduction of multivalent vaccines against both BVD and infectious bovine rhinotracheitis (IBR), it was considered possible that a synergistic effect between the two viruses may have been responsible for the postvaccinal syndrome. The possible role of the vaccine as a stressor in cattle already in the acute stage of infection with a BVDV field strain was also suggested (McKercher et al., 1968). Peter et al. (1967), however, noted that animals that died from pvMD failed to develop antibodies to BVDV but could produce antibodies to IBR virus. Thus, they suggested that cattle succumbing to MD or pvMD were uniquely susceptible to BVDV and that this susceptibility involved a failure of the immune system.

BVDV was recognized as a highly contagious disease that was easily spread from herd to herd (Pritchard, 1963). The original outbreaks in New York were described as explosive in character, in which practically all the animals in a herd came down with the disease within a few days (Olafson et al., 1946). Thus, before a vaccine became available, control procedures were limited to protecting the noninfected herd by prevention of direct and indirect contact with infected animals.

By the mid- and late 1960s, modified-live vaccines comprising the attenuated Oregon C24V and NADL strains were in use (Bittle, 1968; Gutekunst, 1968). The latter strain was isolated in 1962 at the National Animal Disease Laboratory and attenuated in porcine kidney cell culture. Although modified-live vaccines were considered efficacious in preventing acute BVD, their use was thought to be somewhat risky due to the occurrence of pvMD. The risk of pvMD was somewhat a matter of perception and personal experience. Bittle (1968), describing the Oregon C24V vaccine, stated that the incidence of postvaccinal problems reported to the USDA compared to the number of doses used had been extremely small (less than 1 in 10,000) and he encouraged its use. Likewise, Gutekunst (1968) stated that, following 8 months of field use of 350,000 doses of the NADL vaccine, no reports of postvaccinal reactions had been received. In contrast, a commentator at the meeting at which Drs. Bittle and Gutekunst spoke remarked that he had personally observed 40 cases of pvMD in a 3-month period in approximately 1,000 vaccinated cattle (Clark, 1968). Subsequently, it was discovered that pvMD occurs only in persistently infected cattle and although these animals comprise a small portion of the total cattle population, they could comprise a significant portion of animals in an individual herd.

The modified-live vaccines were contraindicated for use in pregnant animals because of the possibility of inducing abortions, and thus, emphasis was placed on vaccination of calves and heifers. Kahrs (1971) also advocated protecting naive cattle from contact with potentially infected cattle, particularly those from auction markets or shipping centers and, probably because of the risk of pvMD, considered this the "best control method." For feedlot cattle, Fuller (1965) stated that it was poor economy to vaccinate already stressed cattle immediately upon arrival to feedlots. Instead, he recommended acclimatizing calves to their new surroundings for about 3 weeks before vaccinating them against BVDV.

THE DECADE OF THE 1970s

PERSISTENT INFECTIONS IN SICK AND APPARENTLY HEALTHY CATTLE

By the 1970s, it was established that calves with congenital BVDV infections were unthrifty and usually died within a few months, and that surviving calves often suffered from chronic disease, were persistently infected with the virus, and were deficient in serum neutralizing antibodies against BVDV (Bürki and Germann, 1964; Malmquist 1968; Johnson and Muscoplat, 1973). These observations were made in sick and unthrifty animals. In 1978, Coria and McClurkin (1978) reported on the persistent infection and immune tolerance of an apparently healthy bull. The bull was continuously viremic and a noncytopathic BVDV was isolated from its blood leukocytes repeatedly from birth to 2.5 years of age. The virus was also isolated repeatedly from the bull's semen. The bull remained seronegative for BVDV antibodies during this time. When challenged with the equivalent of three doses of a killed vaccine, it failed to produce a significant immune response. It was suggested that the bull had acquired the infection early in gestation before the development of its immune system and had thus acquired a specific immune tolerance to BVDV.

A year later, McClurkin et al. (1979) described the reproductive performance of four healthy cattle persistently infected with BVDV: the bull previously identified and three newly identified pregnant cows. As an aid to the identification of apparently healthy, persistently infected cattle in a herd, they noted that cattle that remain seronegative while in contact with seropositive cattle were likely to be immune-tolerant and persistently infected. They further noted that, after three vaccinations of the herd, all cattle except these three cows had serum neutralizing antibody titers of 1:16 or greater against BVDV. As in the persistently infected bull, a noncytopathic BVDV was consistently isolated from the three persistently infected cows, and these cows gave birth to calves that became ill: Two died within the first week postpartum and one calf was euthanized at a few weeks of age. A noncytopathic BVDV was isolated from the blood leukocytes of all three calves indicating maternal transmission.

When seropositive cows were bred by the persistently infected bull, normal calves were born but services per conception averaged a high 2.3. For five seronegative heifers bred by the bull, services per conception averaged 2.0. All seronegative heifers seroconverted to BVDV and had high antibody titers

($\geq 1:128$) 6 weeks after breeding. Four heifers gave birth to normal calves and one heifer aborted at 6 months of gestation but no BVDV was isolated from the fetus. None of the calves produced by either group showed evidence of intrauterine infection. However, the study indicated that BVDV may play a role in repeat breeding problems. It was suggested that losses due to repeat breeding and from neonatal disease might be prevented by ensuring that all cattle have high antibody titers to BVDV before breeding (McClurkin et al., 1979).

Also of interest in this study was the observation that BVDV antibody titers in the herd (38% of the animals had titers $>1:256$) were generally much higher than expected (usually 1:16 to 1:64) for cattle vaccinated with the killed vaccine. It was surmised that virus shedding by the persistently infected animals had constantly challenged the other cattle in the herd and boosted their antibody titers. The idea that the level of BVDV antibodies in a herd could be used to predict which herds contained persistently infected animals was later developed in the 1990s and recently evaluated for herds of unknown BVDV status (Pillars and Grooms, 2002), as will be discussed later in this chapter.

Lesions in the persistently infected, healthy cattle were found to be microscopic, primarily in the brain and kidney (Cutlip et al., 1980). However, immunofluorescence staining of tissues demonstrated widespread distribution of viral antigen in brain and spinal cord neurons, renal glomeruli, renal tubules, lymph nodes, spleen, small intestine crypts, testicular tubules, and endothelial cells.

DIAGNOSIS AND CONTROL

The observation that fetal bovine sera frequently contained BVDV and neutralizing antibodies against BVDV (Kniazeff et al., 1967; Malmquist, 1968) was an increasing concern in the 1970s for cell culture work because contaminated cultures could have undesirable consequences for research and vaccine production. Tamoglia (1968) found that 8% of licensed live IBR vaccines were contaminated with BVDV, raising concerns that such vaccines might give rise to fetal abortions. Experimental studies, like those examining the effects of cytopathic BVDV, could be compromised if cell cultures used to propagate the cytopathic virus were contaminated with noncytopathic BVDV. Commercial fetal bovine sera were contaminated with noncytopathic BVDV as a result of pooling sera from infected and noninfected fetuses. Commonly, sera of 500 fetuses

were pooled and, although the incidence of fetal infection was unknown at the time, Nuttall et al. (1977) calculated that contamination of a batch of fetal bovine serum required only a 0.2% incidence of infection. At the time, the interference test (Gillespie et al., 1962), which relied on the visible inhibition of cytopathic BVDV, was used to screen fetal bovine sera and bovine cells for noncytopathic BVDV. Nuttall et al. (1977) believed that the interference test was not sensitive enough to detect low-level contamination of fetal bovine serum with noncytopathic BVDV and advocated the use of FAT for regular screening of sera and cells for noncytopathic virus.

For serological diagnosis of BVDV infection, Lambert et al. (1974) recommended the use of the serum neutralization assay on paired serum samples collected 2 or 3 weeks apart, with a rising titer indicating active infection. To prevent BVD in neonatal calves, they recommended vaccination of open heifers and cows 30–60 days prior to breeding and the consumption of antibody-rich colostrum by calves.

Because of the concern for pvMD, abortions, and in utero infections, a number of studies were done in the early and mid-1970s on the efficacy of killed vaccines for BVDV. However, these efficacy studies were often limited in scale and involved challenge with homologous virus. Lambert et al. (1971) evaluated a killed BVDV-NADL vaccine and found it efficacious against homologous virus challenge in calves aged 5–11 months. McClurkin et al. (1975) examined the use of killed vaccines using inactivated cytopathic NADL and Singer strains to prevent fetal infection and found these vaccines efficacious. However, these researchers used homologous challenge, and a heterologous or noncytopathic BVDV challenge was not attempted. Challenge with noncytopathic virus would prove to be essential in evaluating fetal protection when it was later discovered that only noncytopathic viruses caused persistent infections.

THE DECADE OF THE 1980s

EXPERIMENTAL PRODUCTION OF PERSISTENT INFECTION AND MUCOSAL DISEASE

In 1984 and 1985, a number of important advances were made in BVDV research. McClurkin et al. (1984) described the production of persistently infected, immune-tolerant calves in five experiments involving 44 cows in the first trimester of pregnancy

(42–125 days). Cows or their fetuses were inoculated directly with one of five different BVDV isolates. Four of the isolates were noncytopathic strains and the fifth was the cytopathic NADL strain. For 38 pregnant cows or fetuses inoculated with noncytopathic strains, there were 10 abortions, 1 stillborn calf, 4 weak or unsteady calves, and 23 calves that had a normal and vigorous appearance at birth. All weak calves and 22 of the 23 normal appearing calves were persistently infected and seronegative and were the result of inoculations occurring at days 42–125 of gestation. One calf infected at 125 days of gestation was immune-competent, virus-negative, and seropositive at birth.

Of interest, but not fully appreciated at the time, was the failure by McClurkin et al. (1984) to produce any persistently infected calves with the cytopathic NADL strain. Prior to this study, Done et al. (1980) had infected 15 pregnant cows at 100 days of gestation with a mixture of 10 cytopathic strains of BVDV. Virus was recovered from eight live-born calves, but in each case these viruses were noncytopathic BVDV. These researchers also did not appear to fully recognize the significance of this finding, stating that “the absence of cytopathogenicity in all reisolates of virus is remarkable, but perhaps no more than a reminder of the genetic variability of viruses in general and of the pestiviruses in particular.” It is almost certain that noncytopathic BVDV contaminated their cytopathic virus stocks used for infection. Later, Brownlie et al. (1989) infected pregnant cattle with a cytopathic virus and could not produce persistent infection. Thus, it became generally accepted that only noncytopathic BVDV could produce persistent infections.

McClurkin et al. (1984) also followed the fate of the persistently infected calves they had produced. All four weak calves either died or were euthanized within 4 months after birth. Of the 22 apparently healthy calves, 6 developed diarrhea and/or pneumonia within 5 months of birth and died, and 1 became unthrifty and remained small. Ten of the apparently healthy calves remained healthy at 6 months of age or as yearlings (most of these calves were then used in other experiments). Three animals remained healthy as 2-year-olds and were bred. Two of the persistently infected cows produced apparently healthy, persistently infected calves, whereas the third cow lost her calf and developed MD at 28 months of age. This study demonstrated several characteristics of persistent infection: that persistently infected calves may be born weak or apparently normal; that some may live to breeding age;

and that persistently infected families can arise by breeding persistently infected cattle.

Soon after the study by McClurkin et al. (1984), Brownlie et al. (1984) and Bolin et al. (1985c) experimentally reproduced MD in persistently infected cattle. Brownlie et al. (1984) noted that while healthy, persistently infected cattle were infected with only noncytopathic BVDV, both noncytopathic and cytopathic BVDV could be isolated from persistently infected cattle that were clinically ill with MD. The latter finding was also observed by McClurkin et al. (1985). From their observations, Brownlie et al. (1984) developed a hypothesis for the induction of MD, which stated that cattle become persistently infected with noncytopathic BVDV after in utero infection and postnatally succumbed to MD when superinfected with a cytopathic BVDV. To test the hypothesis, Brownlie et al. (1984) used a cytopathic isolate from an animal suffering from MD to inoculate two healthy persistently infected herdmates. Both animals came down with MD, supporting the hypothesis. In another study, Bolin et al. (1985c) inoculated persistently infected cattle with noncytopathic or cytopathic BVDV. The cattle inoculated with noncytopathic virus did not develop clinical signs of disease, whereas MD developed in all cattle inoculated with cytopathic BVDV.

Although pvMD was known to be a relatively common phenomenon, failure to consistently induce MD in persistently infected cattle with cytopathic BVDV vaccines (Bolin et al., 1985b) suggested that the induction of MD was somewhat more complicated than simple superinfection with a cytopathic BVDV in an animal persistently infected with noncytopathic BVDV. Subsequent studies indicated that the noncytopathic and cytopathic viruses (called a virus pair) from individual MD cases were antigenically similar. Howard et al. (1987) compared five virus pairs from separate MD outbreaks and found that virus pairs from the same outbreak were antigenically indistinguishable when tested in cross-neutralization tests with antisera. Corapi et al. (1988) using a panel of monoclonal antibodies also showed that noncytopathic and cytopathic viral pairs from MD had a high degree of antigenic similarity. This led to the conclusion that in natural outbreaks of MD the likely origin of the cytopathic BVDV was via mutation of the noncytopathic BVDV infecting the persistently infected animal. Thus, the hypothesis for the induction of MD was refined to include antigenic similarity: MD was induced in a persistently infected animal by “superinfection” with a cy-

topathic BVDV with antigenic similarity to the noncytopathic BVDV. Superinfection could occur by mutation in spontaneous MD or, in the case of pvMD, by vaccination with a vaccine virus antigenically similar to the noncytopathic BVDV infecting the persistently infected animal.

The above hypothesis suggested that inoculation of persistently infected animals with a cytopathic BVDV antigenically different (heterologous) from the noncytopathic persisting virus would not result in MD; but rather, the animal would produce antibodies to the cytopathic virus and clear the infection. This apparently was the reason for the failure of superinfection with cytopathic BVDV (e.g., by vaccination) to produce MD consistently. In support of this hypothesis, Moennig et al. (1990) showed that persistently infected animals superinfected with closely related cytopathic BVDV developed MD within 14 days of infection but those that were superinfected with heterologous cytopathic BVDV did not develop MD within a 2–3-week time frame. Instead, these animals developed antibodies to the superinfecting virus. Other studies, however, showed that the inoculated cytopathic BVDV could sometimes be heterologous and yet precipitate MD in a persistently infected animal. Westenbrink et al. (1989) inoculated 14 clinically healthy, persistently infected animals with three heterologous cytopathic viruses. Twelve of these animals developed MD, some within the expected time frame for MD of 2–3 weeks postinoculation, and several others after several months (so called *late-onset MD*). The actual range when MD began was 17–99 days. Interestingly, neutralizing antibodies were produced against the inoculated cytopathic virus but, as expected, not to the persisting noncytopathic virus. However, in 10 of 12 cases, the neutralizing antibodies did not neutralize the cytopathic virus recovered at necropsy from the intestines. This suggested that mutational changes had occurred and, as a result, cytopathic virus antigenically similar to the persisting noncytopathic virus arose and induced MD.

Shimizu et al. (1989) also produced MD in persistently infected cattle with inoculation of heterologous cytopathic BVDV. Similar to the findings of Westenbrink et al. (1989), they found that the cytopathic viruses recovered from blood early after infection were antigenically similar to the challenge cytopathic virus but that the cytopathic viruses isolated from the carcasses at necropsy were antigenically different from the challenge virus but similar to the noncytopathic persisting virus. Neutralizing antibodies were produced to both the challenge

virus and cytopathic virus isolated early in infection but not to the persisting noncytopathic virus nor to the cytopathic virus recovered from the carcasses at necropsy. These results again suggested that mutational changes or selection of antigenic variants in the challenge virus occurred, which resulted in a homologous cytopathic virus responsible for precipitating MD. However, subsequent studies would show that the arising homologous cytopathic virus was often the result of RNA recombination between the cytopathic challenge virus and the persisting noncytopathic virus.

ACUTE AND PERSISTENT INFECTIONS IN THE BULL

Whitmore et al. (1978) examined BVDV shedding in the semen of nine bulls after acute infection and found detectable virus in 4 of 70 semen samples. They were also able to recover virus from the testicle of one bull. The positive samples were obtained in the first 10 days postinoculation from four of the nine bulls but the amount of virus in the semen was not quantified. Treatment of the bulls with dexamethasone at 28 or 56 days postinoculation did not result in additional virus shedding in semen. More recently, Kirkland et al. (1991) studied five bulls during an acute infection with BVDV. Semen samples were collected between 7 and 14 days after infection on four occasions from each bull. Virus was isolated from three bulls and from 9 of 12 batches of semen from these bulls. Titers of virus were low ranging from 5–75 TCID₅₀/ml of semen. They also noted that acute infection did not appear to affect the quality of semen.

Subsequent to Coria and McClurkin's (1978) report, discussed earlier, Barlow et al. (1986) reported on virus shedding in a persistently infected bull. Virus was shed at high titer ($10^{4.0}$ – $10^{5.5}$ TCID₅₀/0.2 ml) in the semen. This animal had a sight defect, which was discovered postmortem to be due to retinal atrophy, but otherwise the bull appeared normal. Upon examination, the semen had been of acceptable quality and the bull had successfully sired a calf. Thus, had the bull not had a vision defect, the risk it posed of transmitting BVDV might have gone undetected. Revell et al. (1988) reported on two bulls that were persistently infected. In contrast to the findings of Barlow et al. (1986), the quality of the semen of these bulls was consistently poor, as measured by density and motility. Gross abnormalities of sperm heads ("collapsed heads") was seen in 28–45% of spermatozoa from one of the bulls (Cy 105). Paton et al. (1990) inseminated six seronegative and six

preimmunized, seropositive heifers with semen from this bull (Cy 105). Both groups had poor rates of conception but eventually all but one heifer conceived after repeated inseminations. Eleven apparently normal calves were born and none were persistently infected. Paton et al. (1989) also used virus isolated from the serum of Cy 105 to infect four Freisen bulls. Virus was isolated from the semen of one of the bulls from four collections taken 7–14 days postinoculation. The highest titer obtained was $10^{1.4}$ TCID₅₀/ml. Semen quality of the bull was found to deteriorate following infection and showed a reduction in both sperm density and motility.

Meyling and Jensen (1988) were the first to report the production of a persistently infected calf sired from the semen of a persistently infected bull. Twelve seronegative heifers were inseminated with semen from this bull containing 10^5 – $10^{7.5}$ TCID₅₀/ml of BVDV. All 12 heifers became infected as indicated by seroconversion and all heifers gave birth to clinically normal calves. Only 1 of the 12 calves was persistently infected. Later, Kirkland et al. (1994) described the results of widespread field use of semen from a persistently infected bull. Approximately 600 doses of semen had been distributed to 97 dairy farms for sire evaluation purposes and 162 cows were inseminated. The first service conception rate was only 38%. A subsequent study of 61 calves sired by the bull revealed that only two of these calves were persistently infected. From these studies, it is apparent that production of persistently infected offspring via BVDV-contaminated semen is an uncommon event.

RESPIRATORY DISEASE

During the 1980s, research into the contribution of BVDV to respiratory disease was initiated. This research followed the successful reproduction of "shipping fever" pneumonia in cattle with aerosols of infectious bovine rhinotracheitis virus and *Mannheimia haemolytica* in the late 1970s (Jericho and Langford, 1978). Shipping fever pneumonia, a cause of significant mortality in cattle in feedlots, was thought to be precipitated by the stress of transporting cattle from the farm to the feedlot. The pathogenesis of the disease is thought to involve dual infection of a pneumotropic virus and a colonizing bacterial species, most often *M. haemolytica* (Yates, 1982). As early as the first description of BVDV herd outbreaks in 1946, the virus has been implicated in causing at least mild respiratory disease with symptoms of nasal discharge and coughing. Potgieter et al. (1984, 1985) performed several

experiments examining the ability of BVDV to induce respiratory disease. BVDV infection of calves without subsequent bacterial superinfection resulted in mild respiratory tract lesions characterized by small, scattered areas of interstitial pneumonia involving 2–7% of the total lung volume. Infection of calves with *M. haemolytica* alone produced localized lesions involving about 15% of the lung. In contrast to the mild disease produced by these agents when given individually, inoculation of BVDV followed by *M. haemolytica* produced a severe fibrinopurulent bronchopneumonia and pleuritis involving 40–75% of the lung volume. Potgeiter (1985) also found that BVDV strains may differ in their pneumopathogenicity. That BVDV infection can precipitate severe respiratory disease is also supported by the observation that pneumonia, including shipping fever-like fibrinous pneumonia, was a common finding in the severe type 2 BVDV outbreaks that occurred in Ontario, Canada, in the mid-1990s (Carman et al., 1998, van Dreumel, 2002).

THROMBOCYTOPENIA

Thrombocytopenia with hemorrhage associated with BVDV virus infection was first reported in 1987 within a summary of case reports for dairy herds in the northeastern United States (Perdrizet et al., 1987). In one report from 1985, 6 of 30 first-calf heifers developed a high fever, had bloody and mucoid diarrhea, and died within 2 weeks. Rebhun et al. (1989) reviewed case records of cattle admitted to the College of Veterinary Medicine at Cornell University for the years 1977–1987 and found that thrombocytopenia was reported in about 10% of clinically acute BVDV infections in adult cattle. Clinical signs included hemorrhages, red or orange bloody diarrhea, epistaxis, and abnormal bleeding from injection sites. Hemorrhages associated with BVDV infection in young veal calves were also observed with increasing frequency in the late 1980s in the northeastern United States (Corapi et al., 1990b).

Corapi et al. (1989) experimentally reproduced thrombocytopenia in young calves with a BVDV isolate (CD-87) recovered from a severe outbreak. This outbreak involved 50% of a milking herd of 100 holsteins in New York in which 20 animals died. Of the eight calves inoculated with the CD-87 isolate, three developed severe thrombocytopenia ($\leq 5,000$ platelets/ μl). Two of these three calves developed hemorrhages when their platelet counts fell to 2,000/ μl of blood or less for a period of 24 hours or longer. Hemorrhages were observed on the sclera of the eyes, inner surface of the eyelids, mucosal

surfaces of the cheeks, lower gingiva, tongue, and soft palate. Both calves had prolonged bleeding from venepuncture sites. Internal hemorrhages were also observed on the surfaces of various organs of one calf at necropsy. In a later report, Corapi et al. (1990b) inoculated 8 veal calves with the CD-87 isolate and 10 veal calves with CD-89, a BVDV isolate recovered from a veal calf on a farm in Pennsylvania where hemorrhages were noticed in several calves. Ten in-contact calves were included in the experiment. During the experiment, virus was isolated from all calves including in-contact calves. Severe thrombocytopenia was observed in 12 calves, and 11 of these developed hemorrhages. Calves that had a pre-exposure virus-neutralizing antibody titer of $>1:32$ to the Singer strain of BVDV did not develop severe thrombocytopenia. Five calves died during the course of the experiment, four of which exhibited hemorrhages, and the others recovered. Hemorrhages were observed in these experiments when platelet counts decreased below 5,000 platelets/ μl . Although the majority of severely thrombocytopenic calves recovered, there was no way to determine beforehand the calf's fate since those that died often appeared to be in relatively good physical condition just hours prior to death (Corapi et al., 1990b). A few years later, it was discovered that hemorrhagic syndrome, as the disease came to be known, was caused by a new type of BVDV, genetically distinct from the classical viruses used in BVD vaccines.

ADVANCES IN MOLECULAR BIOLOGY

In the late 1980s, significant advances were made in the molecular biology of BVDV with the first genomic sequencing of BVDV strains, the finding of a marker protein for cytopathic BVDV, and the first evidence of RNA recombination in cytopathic strains of BVDV. Monoclonal antibodies to the virus were also first produced in the late 1980s as reagents for protein studies and diagnostic test methods (see the following section, "Diagnosis").

The first BVDV strains to be sequenced were two cytopathic strains, the North American NADL strain and the European Osloss strain (Collett et al., 1988b; Renard et al., 1987). The NADL sequence showed that the RNA genome of BVDV has one long open reading frame (ORF). Thus, proteins are produced by cotranslational and posttranslational processing of a polyprotein (Collett et al., 1988a). The original Osloss sequence was at first shown to contain two ORFs but was later corrected to consist also of a single ORF (de Moerloose et al., 1993).

The elucidation of the genomic organization of BVDV and the pestivirus genus led to their taxonomic reclassification from the *Togaviridae* to the *Flaviviridae*.

At about the same time, protein studies revealed that the two biotypes of the virus, cytopathic and noncytopathic, could be distinguished at a molecular level. It was found that cytopathic strains of BVDV produce in infected cells one additional nonstructural protein not observed in cells infected with noncytopathic BVDV (Donis and Dubovi, 1987; Pocock et al., 1987). The protein, which is a marker protein of cytopathic BVDV, is designated p80 or NS3. This protein is actually a smaller version of a larger nonstructural protein, p125 or NS2-3, that is present in all BVDV-infected cells. Several mechanisms leading to expression of NS3 were later found to occur: The NS3 protein can be generated by proteolytic cleavage of NS2-3 or generated as the result of genetic duplications or deletions in the genomes of some cytopathic BVDVs (Meyers and Thiel, 1996).

Meyers et al. (1989) compared the BVDV Osloss and NADL genomic sequences with that of the pestivirus CSFV and found insertions in the NS2 gene of these cytopathic strains of BVDV. The Osloss insertion was 228 nucleotides long whereas the insertion of NADL was 270 nucleotides. The insertions were found to be different and that of NADL was unidentified. However, remarkably, the Osloss insertion was found to be derived from the cellular gene coding for ubiquitin. These findings led to a molecular model for pathogenesis of MD: In persistently infected animals, the noncytopathic virus mutates to a cytopathic virus by the incorporation of cellular sequences during a recombination event. Subsequently, it was found that genetic recombination could also occur between BVDV genomes as evidenced by insertions of viral sequences in the genomes of some cytopathic viruses.

DIAGNOSIS

Meyling (1984) described a microisolation test for detection of BVDV in serum samples that used immunoperoxidase staining of viral antigens rather than immunofluorescence. The immunoperoxidase monolayer assay (IPMA) is still commonly used today because of its ease and the ability to test many serum samples for BVDV at a time. Prior to the development of the assay for serum, Bielefeldt-Ohmann (1983) had utilized the immunoperoxidase staining technique for the detection of BVDV in tissues of infected animals.

Panels of monoclonal antibodies were produced to both BVDV and CSFV in the late 1980s and early 1990s by several research groups. The specificity of these monoclonal antibodies was usually to the E2 (gp53) protein, which was determined to be the major neutralizing envelope protein of the virus (Donis et al., 1988), or to the nonstructural NS3 (p80) protein or less frequently, to the E^{rns} (gp48) protein, a second envelope protein, which was discovered later to have RNase activity (Schneider et al., 1993). Edwards et al. (1988) examined a total of 38 monoclonal antibodies, of which 26 were against BVDV antigens and 12 were against CSFV antigens. The monoclonal antibodies could be divided into three panels: those that were pan-pestivirus specific; those that were CSFV-specific; and those that were selectively reactive with ruminant pestiviruses. Monoclonal antibodies to the NS3 protein tend to be cross-reactive with all pestiviruses, because the amino acid sequence of this protein is highly conserved among pestiviruses. Monoclonal antibodies to the envelope proteins E2 and E^{rns} tend to be specific for the viral species (i.e., BVDV or CSFV) used as the immunogen in their production (Edwards et al., 1991). Corapi et al. (1990a) examined the cross-reactivity of a panel of BVDV monoclonal antibodies to 70 BVDV isolates. They found that 12 of 13 of their NS3 monoclonal antibodies reacted with 100% of BVDV isolates tested, whereas the reactivity of E2 monoclonal antibodies varied from 6–98%. Of two E^{rns} monoclonal antibodies, one was reactive to 100% of BVDV isolates tested, whereas the other was less cross-reactive (57%). For the 70 BVDV isolates, a total of 32 distinct patterns of monoclonal antibody reactivity were observed. This demonstrated that considerable antigenic diversity exists among BVDV isolates.

CONTROL

To gain insight into the carrier state and for control strategies for BVDV, it was important to determine the prevalence of persistently infected cattle in entire populations. Meyling (1984), used the immunoperoxidase monolayer assay and found that approximately 1% of slaughter cattle in Denmark were viremic and apparently persistently infected. Subsequently, Bolin et al. (1985a) made the first attempt to determine the prevalence of persistently infected cattle in U.S. herds. The prevalence of persistent infection in a nonrandom population of 66 herds was 1.7%. Since 50% of these herds were chosen because of a history of BVDV infection, it was noted that the prevalence figure for the entire U.S. cattle

population was probably somewhat lower. However, the figure of 1.7% turned out to be similar to the prevalence of persistently infected cattle of approximately 0.5% to 2.0% obtained in later surveys conducted in different countries in the 1980s and 1990s (Houe, 1999). In their study on the prevalence of persistently infected cattle, Bolin et al. (1985a) also titrated the virus from the serum of persistently infected animals and found that these animals usually had high BVDV titers (10^4 – 10^5 TCID₅₀/ml).

At about the same time as the study of McClurkin et al. (1984) on the production of immune-tolerant, persistently infected calves, Liess et al. (1984) produced congenital malformations (cerebellar hypoplasia, hydrocephalus) and persistent infections in calves by inoculation of pregnant cattle with a modified-live vaccine and demonstrated the risk of using these vaccines in breeding animals. In both studies, persistently infected calves could be produced by inoculation of pregnant cows to about 120 days of gestation, after which BVDV infection generated a fetal immune response.

By the late 1980s, modified-live vaccines, a temperature-sensitive mutant virus vaccine (Lobman et al., 1984), and killed-virus vaccines were available for use. Two elements of control were considered essential: the detection and elimination of persistently infected carriers and immunization of breeding animals before their first conception (Radostits and Littlejohns, 1988). Immunization of calves was now considered to be less important by some. Previously, before the pathogenesis of MD was understood, it was thought by some that immunization of young cattle might prevent MD from occurring because the disease usually occurs in cattle from 6–24 months of age. Radostits and Littlejohns (1988) commented that pvMD gave vaccines a poor reputation and as a result they had not been used on a regular basis. There was also a concern that modified-live BVDV vaccines might cause immunosuppression and increase the risk of mortality in feedlot cattle (Martin et al., 1980; 1981).

Radostits and Littlejohns (1988) stated that there was no substantial evidence to warrant the vaccination of feedlot cattle. They further suggested that, if vaccination of the dam prior to conception is a part of the control program, vaccination of calves may be unnecessary until they approach breeding age. Thus, there was a de-emphasis of vaccination for some groups of cattle (calves and feedlot animals), at least in some circles. Baker (1987), cited studies showing an association between BVDV vaccination and increased risk of mortality in feedlot animals and a re-

port on the immunosuppressive properties of a modified-live (Singer strain) vaccine (Roth and Kaerberle, 1983) and believed that it would be advantageous to vaccinate calves in a preconditioning program before their arrival at a feedlot. If calves are vaccinated on arrival at feedlots, he believed they should be vaccinated with a killed-virus vaccine.

Bolin (1990) also stated the concerns regarding the use of modified-live vaccines: immunosuppression, the potential to produce pvMD, and the potential to adversely affect the fetus. During the late 1980s, there was also a growing awareness of the antigenic diversity among BVDV isolates. Thus, another concern for vaccines in general was their efficacy in protecting against fetal infections with antigenically variable field viruses. Bolin (1990) recommended using modified-live vaccines in large grazing herds or when handling facilities for cattle were poor, since only a single dose of vaccine is required for immunization. Modified-live vaccines were contraindicated for use in pregnant cattle and animals in contact with pregnant cattle. Instead, Bolin (1990) recommended the use of killed-virus vaccines in dairy herds where pregnant cattle are always present. Killed-virus vaccines were also recommended for bulls in semen collection centers.

Although the identification and elimination of persistently infected cattle was considered an essential element of control, it was also considered costly because of the amount of testing involved. Baker (1987) recommended testing for virus or viral antigen and determining antibody status, but stated that screening of whole herds may not be economically feasible in all situations. He suggested an alternate approach to reduce the cost of testing. Persistently infected animals are viremic, usually antibody-negative, and often exhibit a poor response to vaccination. Hence, one possibility is to vaccinate all cattle greater than 6 months of age with a killed-virus vaccine followed by a booster, and then determine their antibody titers. Cattle that remained antibody-negative or respond poorly with low levels of antibody would be suspected to be persistently infected and could be tested for virus or viral antigen. This was reminiscent of the observations of McClurkin et al. (1979) that cattle that remain seronegative while in contact with seropositive cattle are likely to be immune-tolerant and persistently infected, and these cattle had low antibody titers after vaccination. However, because the immune tolerance is specific for the persisting BVDV in these animals, screening by this method may not be highly reliable, particularly when the vaccine is antigenically very different

from the virus persisting in the herd. In the latter case, higher than expected levels of antibodies might be produced by persistently infected animals and obscure differences in antibody levels between persistently infected and normal cattle.

Baker (1987) noted several important factors that should be considered in herd screening, including that persistently infected calves may be seropositive because of colostral antibodies, and that passive immunity may interfere with virus isolation. Furthermore, any calves born in the 9 months after herd testing potentially may be persistently infected. He also noted that retesting along family lines may be worthwhile because of the possibility of persistently infected families. After a herd is free of persistently infected animals, he recommended isolating and testing all new additions to the herd and that any additions that are pregnant should also have their calves tested for virus at birth.

THE DECADE OF THE 1990s

THE EMERGENCE OF TYPE 2 BVDV

In the early and mid-1990s, in addition to outbreaks of hemorrhagic syndrome (severe thrombocytopenia with hemorrhages) (Ridpath et al., 1994), outbreaks of acute, severe BVD in which hemorrhagic syndrome was either inapparent or was not a prominent clinical feature, occurred in Canada and the United States (Pellerin et al., 1994; Carman et al., 1998; Sockett et al., 1996; Drake et al., 1996). In Canada, these BVD outbreaks were most damaging to the cattle industry in the provinces of Quebec and Ontario. In 1993, Quebec lost approximately 25% of its veal crop (overall mortality: 32,000 out of 143,000 calves) to these BVDV outbreaks (Pellerin et al., 1994). Although the Quebec outbreaks were not described in detail from a clinical standpoint, Pellerin et al. (1994) stated that herds of calves that looked healthy one day could suffer a 10% death loss the next day, and it was not a rarity to have a mortality rate of 100%. Fever, pneumonia, diarrhea, and sudden death occurred in all age groups and abortions were frequent in the Ontario outbreaks of 1993 to 1995 (Carman et al., 1998). The disease often resembled MD. However, severe acute BVD could be distinguished from MD because only non-cytopathic BVDV was isolated from animals suffering severe acute BVD.

Pellerin et al. (1994) and Ridpath et al. (1994) determined that the BVDV isolates causing hemorrhagic syndrome and acute severe BVD formed a new genetic group (genotype) of BVDV distinct

from early strains such as the Oregon C24V, NADL, and Singer strains utilized in vaccines. The new group was designated type 2 (BVDV 2) and the group comprising the early strains as type 1 (BVDV 1). Pellerin et al. (1994) further subdivided BVDV 1 into two subgroups: 1a, comprising such strains as NADL, Oregon, and Singer; and 1b, which included NY-1 and Osloss strains.

In the Ontario BVDV 2 outbreaks, the initial clinical complaint was frequently of respiratory disease in calves or adults (Carman et al., 1998). Diarrhea and abortion were also listed as initial clinical signs. Postmortem lesions were generally those described for MD: gastrointestinal erosions and ulcers. Pneumonia was the most common concurrent diagnosis and was observed in all age groups.

Retrospective typing of Ontario isolates recovered from 1981–1994 proved that as early as 1981, BVDV 2 was already present in Ontario, Canada (Carman et al., 1998). Since outbreaks of severe acute BVD did not occur until much later (1993), this suggests that either earlier circulating strains of BVDV 2 were not highly virulent and that some strains had subsequently acquired virulence determinants or, alternatively, virulent strains of BVDV 2 existed early but were harbored in seropositive herds and only later caused outbreaks of severe disease when naive populations became exposed and infected.

PHYLOGENETIC STUDIES

Prior to the decade of the 1990s, BVDV, CSFV, and BDV were the three recognized pestiviruses, although it was uncertain whether BDV represented a unique viral species or whether border disease in sheep was caused by BVDV. During the 1990s, in addition to the segregation of BVDV into two genotypes, genetic characterization of isolates from sheep showed that sheep could be infected by both genotypes of BVDV and by a unique pestivirus that was referred to as the “true” border disease virus (Becher et al., 1995). The first “BDV” isolate sequenced was actually a BVDV 2 strain (Sullivan et al., 1994; Becher et al., 1995). It was also demonstrated that wildlife could be infected with pestiviruses. Genetic analysis of a pestivirus isolated from a giraffe proved this virus to be a unique genotype, whereas three deer isolates were found to belong to the BVDV 1 genotype (van Rijn et al., 1997; Becher et al., 1997).

Baule et al. (1997) reported that southern African isolates consisted of four BVDV 1 subtypes, including the subtypes 1a and 1b previously described

(Pellerin et al., 1994; Ridpath et al., 1994). The two new BVDV 1 subtypes from southern Africa were designated 1c and 1d. The latter BVDV subtype was reported to be predominantly associated with respiratory tract disease and later shown experimentally to be able to cause primary respiratory disease (Baule et al., 2001). Vilček et al. (2001) examined 76 BVDV 1 isolates from various countries and reported that these viruses could be separated into 11 phylogenetic groups. Recently, Flores et al. (2002) identified six South American strains and one North American BVDV 2 strain that cluster in a separate group from other BVDV 2. Thus, two subgenotypes of BVDV 2 (2a and 2b) have now been identified.

GENETIC RECOMBINATION AND SPONTANEOUS AND POSTVACCINAL MUCOSAL DISEASE

After the findings by Meyers et al. (1989) of the cellular insertion of ubiquitin-coding sequences in the NS2 gene of cytopathic Osloss strain of BVDV and another unidentified insertion (also identified later to be of cellular origin) in the same gene of the NADL strain, much of the research activity in the early and mid-1990s was devoted to the genetic analysis of cytopathic and noncytopathic virus pairs from cases of MD. This analysis revealed a number of genetic changes in cytopathic BVDV, including insertions and gene duplications and deletions, changes that resulted in the production of NS3 by creation of a cleavage site at its amino terminus (reviewed by Meyers and Thiel, 1996). In cases where viral genes were duplicated, an additional NS3 gene generated the NS3 protein. The genetic changes observed in cytopathic BVDV demonstrated that both viral-cellular and viral-viral recombination were the operating mechanisms for the generation of cytopathic BVDV from the persisting noncytopathic BVDV.

In 1995, Ridpath and Bolin (1995) characterized a noncytopathic (BVDV 2-125nc) and cytopathic (BVDV 2-125c) viral pair isolated from an animal suffering from late-onset pvMD. This animal had been vaccinated 3 months prior with a modified-live BVDV 1 (NADL strain) vaccine. Genetic sequencing of the BVDV 2 viral pair revealed that in comparison with BVDV 2-125nc, the BVDV 2-125c isolate contained a 366-nucleotide insertion in the NS2-3 gene (leading to the expression of the NS3 protein). The inserted sequence was found to have a 99% identity with sequences of the BVDV 1-NADL vaccine virus, indicating that the vaccine virus had recombined with the noncytopathic virus BVDV

2-125nc to produce the cytopathic BVDV 2-125c. The authors speculated that the prolonged time between vaccination and pvMD may have been a reflection of the time required for a single virus particle, generated by the recombination event, to replicate to a high enough titer to precipitate pvMD. The cytopathic BVDV 2-125c was one of the first isolated and characterized cytopathic BVDV 2 strains and has since been used by many laboratories as a challenge virus in serum neutralization assays for determination of neutralizing antibodies to BVDV 2.

Subsequently, Becher et al. (2001) also found, in two independent cases of pvMD, that genetic recombination between the persisting noncytopathic BVDV and a BVDV vaccine virus had occurred. In one of these cases, the cytopathic component of the virus pair was shown to have been generated by both recombination and deletion events and was actually comprised of five different cytopathic BVDV subgenomes (genomic BVDV RNA containing large internal deletions but able to express the NS3 protein).

LATE-ONSET MUCOSAL DISEASE

Several experimental studies were conducted on late-onset (or *delayed-onset*) MD in the mid- and late 1990s. Whereas in MD (or acute MD) the superinfecting cytopathic BVDV kills the persistently infected host within about 3 weeks, in late-onset MD there may be several months between challenge with the cytopathic virus and the onset of clinical signs of disease. Late-onset MD was first described by Brownlie et al. (1986), who infected six persistently infected calves with homologous cytopathic BVDV and six with heterologous cytopathic BVDV. All six animals superinfected with the homologous virus developed MD in the first 3 weeks, whereas none of the animals superinfected with a heterologous virus developed MD in this time period. However, two of the animals infected with the heterologous virus developed late-onset MD at 98 and 146 days postinoculation. Three others remained healthy until euthanized at 59–209 days postinoculation. The sixth animal appeared to be developing late-onset MD at about 80 days with signs of intermittent diarrhea over a 4-week period. Later, Westenbrink et al. (1989) also inoculated clinically healthy, persistently infected animals with three heterologous cytopathic BVDV, and MD occurred as late as 99 days postinoculation. It should be noted here that since spontaneous MD can occur in persistently infected animals that are not superinfected and are held in isolation (Brownlie and Clarke, 1993), it is possible

that some experimental cases of late-onset MD (and pvMD), could actually be cases of spontaneous MD. Thus, in experiments of this type, an analysis is needed (preferably genetic sequence analysis), beyond that of virus-neutralizing antibody responses, of the superinfecting *input* cytopathic BVDV, the persisting noncytopathic virus, and the *output* or MD-associated cytopathic BVDV.

Moennig et al. (1993) inoculated a persistently infected bull with a heterologous cytopathic BVDV (TGAC), and MD was observed 15 weeks after inoculation. In this case, the neutralizing antibody response to the cytopathic TGAC virus was delayed 31 days postinfection and neutralizing antibody titers increased to a maximum on day 114 postinoculation (when the animal was euthanized), indicating prolonged persistence of the superinfecting cytopathic virus in this animal. However, only noncytopathic virus was recovered from buffy coat cells and nasal swabs. The cytopathic BVDV recovered from fecal samples (designated cpX) at the onset of disease was analyzed and found to have the same phenotype (monoclonal antibody reactivity pattern) as the noncytopathic persisting virus but the same genotype in the NS2-3 region as the TGAC cytopathic virus as observed by restriction enzyme digestion of PCR products (Fritzemeier et al., 1995). Thus, they concluded that cpX was a phenotypically altered variant of TGAC. Subsequent nucleotide sequencing (Fritzemeier et al., 1997) indicated that cpX arose by recombination of persisting noncytopathic virus and TGAC. This finding was consistent with the finding of Ridpath and Bolin (1995) of viral-viral recombination for a case of late-onset pvMD. Fritzemeier et al. (1997) also found that a superinfecting cytopathic BVDV in experimental *acute* MD (MD occurring in a 2–3-week time frame after inoculation) remained genetically unchanged from inoculation to the onset of MD. They concluded that acute MD and late-onset MD may occur by two different mechanisms: In the former, it is the superinfecting virus that causes the disease; whereas in the latter, genetic recombination between the superinfecting cytopathic virus and the persisting noncytopathic virus creates a new cytopathic virus that causes the disease. Subsequently, Fray et al. (1998) inoculated a persistently infected calf with culture of an uncloned heterologous cytopathic BVDV obtained from a natural case of MD (thus also containing a noncytopathic virus) and the animal developed MD at 145 days postinoculation. Of interest in this study was the occurrence of a prolonged nasal shedding and viremia of cytopathic BVDV before the

onset of MD. No genetic analysis of viral isolates was conducted, but neutralizing antibody data indicated that at least three antigenically distinct cytopathic viruses were isolated from the calf during the period between superinfection and postmortem examination. The authors speculated that the noncytopathic virus in the heterologous challenge may have influenced the survival of cytopathic BVDV in the animal in some manner, possibly by influencing the immune response of the host.

NORMAL CALVES FROM PERSISTENTLY INFECTED COWS

Calves born to persistently infected cows are likewise persistently infected; thus these animals are unsuitable for breeding (McClurkin et al., 1984). Wentink et al. (1991), in an attempt to preserve the genetic material of a highly valued heifer that was developmentally normal but persistently infected, transferred a fertilized embryo of the heifer to an immunocompetent recipient cow. Before transfer, the embryo was treated and washed according to routine methods of the International Embryo Transfer Society (IETS). The result was a normal heifer calf with normal immunity to BVDV. Since then, several other researchers have repeated these results and have produced, by embryo transfer, normal calves from persistently infected cows (Bak et al., 1992; Brock et al., 1997; Smith and Grimmer, 2000).

It was noted however, that there was a lack of a superovulatory response following hormone stimulation in persistently infected cows and only low numbers of viable embryos could be obtained. Wentink et al. (1991) obtained only one viable embryo out of six, and Brock et al. (1997), who used seven persistently infected donors, obtained only nine transferable embryos from two of the females after 45 individual uterine flushes. In the study of Brock et al. (1997), only one pregnancy was obtained from the transfer of six embryos into seronegative recipients. None of the recipient cows showed seroconversion to BVDV leading the authors to conclude that neither horizontal nor vertical transmission of the virus occurs when recommended IETS embryo washing procedures are followed.

ATYPICAL PERSISTENT INFECTION

In 1998, Voges et al. (1998) described for the first time a persistent infection of BVDV in an immunocompetent animal. The bull in this case was not viremic but continuously shed virus in its semen over a period of 11 months until slaughtered. The bull ap-

peared healthy and its growth rate and testicular development were unremarkable. Semen quality was considered good. Limited pregnancy data suggested that the *in vivo* fertility of the bull's sperm was not compromised. The animal had a consistently high titer of serum neutralizing antibodies to the standard test virus ($\geq 1:4096$). Against the homologous virus, the animal had an extraordinarily high serum neutralizing antibody titer of $>1:100,000$.

The virus titer in the semen was relatively low ($<10^{3.3}$ TCID₅₀/ml) when compared to semen obtained from typical, persistently infected (viremic) bulls (approximately 10^5 – 10^7 TCID₅₀/ml) (Barlow et al., 1986; Meyling and Jensen, 1988; Kirkland et al., 1991). On postmortem, BVDV was isolated from testicular tissues but not from any other tissue examined. This long-term shedding of BVDV in a non-viremic bull is reminiscent of the situation in stallions infected with equine arteritis virus (Timoney and McCollum, 1993). In this infection, stallions undergo a transient viremia resulting in an immune response that eliminates the virus except for that in the genital tract. Chronic shedding of the virus in the semen occurs in many infected stallions and may persist for years, although most horses eventually clear the infection. These carrier stallions also have high serum neutralizing antibody titers.

As hypothesized by Voges et al. (1998), the bull with the atypical persistent infection was likely infected near the age of puberty, shortly before the blood-testis barrier becomes fully functional. This allowed infection of testicular tissues but excluded ensuing antibodies from the site. The high level of serum neutralizing antibodies suggests continual exposure of the immune system to the virus. This atypical persistent infection of BVDV in bulls appears to be rare, but at least one other case has been reported (van Rijn, 1999). Nevertheless, this has changed the testing requirements for bulls entering artificial insemination (AI) centers. Previously, testing for viremia was all that was required to identify typical persistently infected bulls and to restrict their entry; semen was rarely tested. Now the World Organization for Animal Health (Office International des Epizooties) requires the testing of semen from each bull before entry into an AI center.

ADVANCES IN MOLECULAR BIOLOGY

In the latter half of the 1990s, infectious cDNA clones were constructed for the type 1, cytopathic BVDV strains cp7, and NADL (Meyers et al., 1996; Vassilev et al., 1997; Mendez et al., 1998). Subsequently, 40 years after its initial discovery (Gillespie

et al., 1960), an infectious cDNA clone was constructed from the genome of the type 1, cytopathic Oregon strain (Kümmerer and Meyers, 2000). The availability of infectious cDNA clones has begun to have, and will continue to have, a large impact on our understanding of the molecular biology of BVDV as these cDNA clones are manipulated and used in studies to decipher the function of genes and genetic elements.

Meyers et al. (1996) removed an insertion of 27 nucleotides in the NS2 gene of the infectious clone of the cytopathic cp7, which is not present in the genome of cp7's noncytopathic counterpart, ncp7 (the two viruses representing a homologous viral pair from a case of MD). In removing the insertion, a noncytopathic BVDV was recovered, proving that the insertion was responsible for the cytopathogenicity of the cp7 virus. Similarly, Mendez et al. (1998) deleted a 270-nucleotide insertion in the NS2 gene of the infectious clone of NADL and also produced a virus that was no longer cytopathic. In both cases, the altered infectious clones of cp7 and NADL failed to produce NS3, showing that these insertions led to processing at the NS2/NS3 site, production of NS3, and cytopathic effect.

Some strains of cytopathic BVDV, such as the Oregon and Singer strains, do not contain insertions or genetic rearrangements and, thus, did not arise by recombination but by another mechanism. Kümmerer et al. (1998) and Kümmerer and Meyers (2000) used chimeric cDNA constructs in a transient expression system and alterations in an infectious cDNA clone to show that processing at the NS2/NS3 site in the Oregon strain was the result of point mutations within the NS2 protein. They noted that about 40 cytopathic pestiviruses have thus far been analyzed and the majority of these have been generated by recombination. The remaining strains have apparently arisen by point mutations generated during replication. However, since these cytopathic viruses occur less frequently than those that arise by recombination (itself a rare event) these researchers hypothesized that this second mechanism of generating cytopathic BVDV was not one of sequential accumulation of point mutations, but rather the simultaneous introduction of a set of point mutations.

Recently, an infectious cDNA clone for a virulent, noncytopathic type 2 BVDV (New York 93 strain) was constructed (Meyer et al., 2002). Infectious virus derived from the cDNA clone retained virulence when used to infect cattle. These animals developed fever, leukopenia, and clinical signs, including respiratory symptoms and gastrointestinal

disorders. Alteration of single histidine codons (at positions 300 and 349) in the sequence of the E^{rns} gene in the infectious cDNA clone led to two new infectious clones with RNase-negative phenotypes. Virus derived from the infectious clone having a deletion of the histidine codon at position 349 was used in animal experiments and was found to have an attenuated phenotype. None of the calves infected with this mutant virus had body temperatures above 39.5°C, nor did they develop diarrhea; only mild respiratory signs were observed. Leukopenia occurred but with an early recovery of leukocyte numbers. Viremia and nasal shedding also occurred but for a shorter duration than in animals infected with virus derived from the unaltered infectious cDNA clone. As demonstrated by this study, the availability of an infectious cDNA clone in which virulent BVDV can be recovered allows new approaches to the study of BVDV-induced disease and identification of genetic markers of virulence and attenuation.

DIAGNOSIS BY VIRUS ISOLATION

An essential element of control of BVDV in herds is the detection and elimination of persistently infected calves. The classical method of detecting persistent infections is virus isolation in cell culture using serum or leukocytes as the test sample. In testing young calves, a major concern with the use of virus isolation, especially from serum, was the presence of colostral antibodies to BVDV, which could interfere with the sensitivity of the test. Palfi et al. (1993) studied the decline of BVDV colostral antibodies and the detectability of BVDV in young, persistently infected calves and found that viremia was not detectable in the serum of seven persistently infected calves with colostral antibody titers, which were as low as 1:16 to 1:24.

In a later study, only one of four persistently infected calves, which had the highest viral titer ($10^{6.5}$ TCID₅₀/ml) before ingestion of colostrum, was detected as viremic after colostrum ingestion when both serum and leukocytes were tested (Brock et al., 1998). Palfi et al. (1993) found that the half-life of colostral antibodies in persistently infected calves (5 to 11 days) was much shorter than in non-persistently infected calves (approximately 3 weeks). Presumably, the continuous high virus production in persistently infected calves is responsible for the rapid clearance of colostral antibodies. Palfi et al. (1993) found that clearance of these antibodies in persistently infected calves may occur by 8 weeks of age, at which time viremia can be detected.

However, it has been generally recommended that, if calves under 3 months of age have been tested by virus isolation, they be retested at 3 months of age (Brock et al., 1998; Saliki et al., 1997). Dubovi (2002) recommended that, for young calves, in addition to the standard washing of leukocytes to remove colostral antibody, fresh, rather than freeze-thawed, leukocytes be used for virus isolation since interference, presumably by residual antibodies, may occur upon freeze-thawing.

Although persistently infected cattle generally have very high BVDV titers (10^4 – 10^5 TCID₅₀/ml of serum), Brock et al. (1998) found that the level of viremia in one of seven persistently infected animals became undetectable over several test dates when serum was tested. In this apparently rare case, the animal developed neutralizing antibodies to the persisting virus although the infection was never cleared. During periods when the virus was undetectable in serum because of neutralizing antibody, virus was isolated at low concentrations from blood leukocytes.

Rae et al. (1987) examined the viability of BVDV in serum and plasma collected from persistently infected cattle. Storage of samples in the dark at room temperature (17–26°C) for up to 5 days had no significant effect on virus titer. They concluded that successful isolation of BVDV from persistently infected animals was unlikely to be compromised by a delay of up to 5 days between sample collection and testing.

MONOCLONAL ANTIBODY-BASED TESTS

With the emergence of BVDV 2, existing panels of BVDV 1 monoclonal antibodies were examined for cross-reactivity with these new isolates, and monoclonal antibodies to BVDV 2 were also produced. Ridpath et al. (1994) tested 29 E2-specific monoclonal antibodies produced against BVDV 1 strains and found that most of these failed to react with BVDV 2 isolates. However, two E2-specific monoclonal antibodies were reactive with all 15 BVDV 2 isolates tested. Deregt and Prins (1998) also determined that one BVDV 1 E2-specific monoclonal antibody was reactive to all 21 BVDV 2 isolates tested, whereas another E2-specific monoclonal antibody was determined to be BVDV 1-specific. The epitopes of these two monoclonal antibodies were mapped, the first to an immunodominant, type-common epitope (Paton et al., 1992; Deregt et al., 1998a) and the latter to a conformational epitope containing a critical amino acid deleted in BVDV 2 isolates (Deregt et al., 1998a). E2-specific monoclonal antibodies

against BVDV 2 that were reactive with neutralizing epitopes in three antigenic domains were also produced (Deregt et al., 1998b). Most of these monoclonal antibodies were unreactive or only weakly reactive with BVDV 1. One monoclonal antibody had a type-common specificity, and two others were entirely BVDV 2-specific.

A number of monoclonal antibody-based tests were developed in the 1990s. Competitive (blocking) enzyme-linked immunosorbent assays (ELISAs) for the detection of antibodies to BVDV were constructed using NS3 monoclonal antibodies (Lecomte et al., 1990; Paton et al., 1991). Paton et al. (1991) used two NS3 monoclonal antibodies reactive to 157 different pestiviruses for development of a blocking ELISA. They examined the ability of the assay to detect antibodies against pestiviruses in cattle, sheep, and swine. The relative sensitivity of the blocking ELISA compared to serum neutralization was high for bovine and ovine sera (94.7% and 99.1%, respectively) but lower for swine (76%), whereas the specificity was high in each case ($\geq 96\%$).

Several capture ELISAs for detection of the NS2-3 protein in persistently infected cattle were also developed and commercialized (Brinkhof et al., 1996). The NS3/NS2-3 proteins are highly immunogenic and the NS2-3 protein is produced in large amounts in persistently infected cattle. Brinkhof et al. (1996) evaluated four commercial capture ELISAs employing NS3 monoclonal antibodies in which blood leukocyte preparations were tested. These assays demonstrated high sensitivity (94–97%) and specificity (100%). The capture ELISA was considered to be the test of choice for eradication programs where many animals need to be screened and monitored. In the above study and in another study (Shannon et al., 1992), false negative results were obtained with capture ELISAs for calves with high levels of colostral antibodies in their blood. Thus, as for virus isolation, it was recommended that if young calves under 3 months of age are tested by capture ELISA for persistent infections they be retested again at 3 months of age.

Haines et al. (1992) developed a monoclonal antibody-based immunohistochemical method for detecting BVDV antigen in formalin-fixed tissues using the monoclonal antibody, 15C5. This monoclonal antibody, with specificity for the E^{ns} protein, was the only monoclonal antibody out of 32 tested that was reactive against antigen preserved in these tissues, suggesting that most protein epitopes of BVDV are conformational in nature. The mono-

clonal antibody 15C5 was found to be particularly useful for this application because its epitope was shown to be highly conserved, reacting with all of the 70 BVDV isolates tested (Corapi et al., 1990a).

Pooled monoclonal antibodies were employed in immunoperoxidase monolayer assays (IPMA) (Saliki et al., 1997; Deregt and Prins, 1998) as well as in a monolayer ELISA (m-ELISA) for microtiter virus isolation (Saliki et al., 1997). Saliki et al. (1997) utilized the E^{ns} monoclonal antibody 15C5 and a NS3 monoclonal antibody, whereas Deregt and Prins (1998) utilized a pool of E2 and NS3 monoclonal antibodies for detection of BVDV 1 and BVDV 2. The m-ELISA uses a spectrophotometer for reading samples and is a more rapid test than the IPMA. The microtiter IPMA and m-ELISA, compared to conventional virus isolation, were shown to have a relative sensitivity of 100% for samples from cattle greater than 3 months of age suspected of being persistently infected and 85% when samples of cattle with acute infections were included (Saliki et al., 1997).

Flow cytometry has also been investigated for use as a diagnostic assay. Qvist et al. (1990, 1991) evaluated its use in identification of persistently infected cattle and found it to be more sensitive than virus isolation. Using a fluorescence-activated cell sorter to analyze fluorescent antibody-bound, infected leukocytes, BVDV-specific antigens were shown to occur in 3–21% (mean 11%) of mononuclear leukocytes of persistently-infected cattle.

POLYMERASE CHAIN REACTION

The first of many reverse transcription-polymerase chain reaction (RT-PCR) assays for BVDV was developed in 1990 (Schroeder and Balassu-Chan, 1990). Although validation of this assay was limited, it was reported to be as analytically sensitive as the IPMA for virus detection. Since the development of this assay, many more sophisticated PCR assays were developed for BVDV in the 1990s, which matched the development of PCR in general for other viruses. These included nested PCR assays with two rounds of PCR to increase analytical sensitivity, multiplex assays with species-specific primers for genotyping, and real-time PCR assays that utilize fluorescent signals from oligonucleotide probes to detect amplification.

With the discovery of BVDV 2 and the existence of two genotypes of BVDV in the mid 1990s, several PCR-based assays for typing BVDV were developed. Harpin et al. (1995) used an indirect method, following RT-PCR with restriction endonu-

clease digestion of the PCR product for typing. Ridpath et al. (1994) utilized the specific amplification of BVDV 2 for typing, with a negative result indicating BVDV 1. Subsequently, these researchers made improvements to their PCR assays, including a positive test for BVDV 1 and specific primers for the differentiation of BVDV 1 subtypes, 1a and 1b (Ridpath and Bolin, 1998). The earlier PCR assays were followed by two multiplex PCR assays in which specific primers for BVDV 1 and BVDV 2 are used in a nested PCR format in the second round of amplification (Sullivan and Akkina, 1995; Gilbert et al., 1999). The advantage of the multiplex assay is that a specific product of differing size is produced for each genotype and differentiation occurs in a single assay. The assay of Sullivan and Akkina (1995) could type BDV as well as BVDV 1 and BVDV 2 since BDV-specific primers were included in the assay. This is seen as an advantage for typing pestiviruses from sheep, which can be infected by all three viruses, but in cattle BDV does not appear to be readily infectious. BDV has not been isolated from North American or European cattle (Paton et al., 1996; Ridpath, 1996) and only one bovine BDV isolate has ever been reported; its original isolation was thought to have been made in the 1960s (Becher et al., 1997). The PCR assay of Gilbert et al. (1999), further described in Deregt et al. (2002) could be used with conventional RNA extraction, or directly without RNA extraction, by adding the sample to the RT-PCR mixture.

Radwan et al. (1995) developed a PCR assay for testing bulk milk samples to identify dairy herds infected with BVDV. For validation of the assay, these researchers first determined the BVDV titers in milk from an experimentally (acutely) infected cow and two persistently infected cows. Virus titers of $10^{2.5}$, $10^{6.5}$, and $10^{5.5}$ TCID₅₀/ml were present in milk from the acutely infected cow and persistently infected cows, respectively. The virus titers in the milk of persistently infected cows were higher than those in their sera (approximately $10^{4.5}$ TCID₅₀/ml). The PCR assay was found to be about 15 times more sensitive than virus isolation in detecting BVDV in milk somatic cells. By testing bulk milk samples, BVDV was detected in 33 of 136 dairy herds by the PCR assay. In sharp contrast, virus isolation did not detect BVDV in any of the bulk milk samples. It was suggested that BVDV antibodies in the milk were responsible for the poor virus isolation results.

Drew et al. (1999) also applied PCR to bulk milk samples to identify BVDV infection among lactating cows, but did not attempt virus isolation from

these samples. Their assay was able to detect BVDV shed by 1 persistently infected cow in a herd of 162 lactating animals. Later, Renshaw et al. (2000) used a PCR assay, which employed the primers used in the assay of Radwan et al. (1995), to detect BVDV in bulk milk samples and also attempted virus isolation from these samples. They found that BVDV could be detected by both methods when milk from a single persistently infected animal was diluted 1:600 with milk from a herd of BVDV-negative animals. Of 144 bulk milk samples from 97 farms, 24 were BVDV-positive by either PCR or virus isolation: 20 were positive by PCR and 17 were positive by virus isolation. Renshaw et al. (2000) suggested that the poor virus isolation rate of Radwan et al. (1995) may have resulted from using milk somatic cells that were frozen before virus isolation was attempted, and that successful virus isolation could be obtained when freshly prepared (not frozen) milk somatic cells are utilized. Furthermore, they recommended simultaneous PCR and virus isolation testing of bulk milk samples to ensure detecting BVDV that may either not be amplified by primers currently in use or which for unknown reasons may be difficult to isolate.

McGoldrick et al. (1999) designed a real-time (Taqman) nested PCR assay for CSFV, which could be modified with different fluorescent probes to allow specific detection of BVDV 2 or BDV. Another fluorescent probe allowed detection of all pestiviruses except for some isolates of BVDV 2. The nested PCR assay was performed in a closed, single tube in which reagents for the second round of PCR were maintained in the inner lid. After reverse transcription and a primary round of PCR, the tubes were inverted to mix the second round reagents in the lid with the first round products for initiation of the second round of PCR. Later, Mahlum et al. (2002) designed a real-time (Taqman) PCR assay specifically for BVDV. The assay was found to be more sensitive than virus isolation and IPMA in detecting BVDV in sera, and more sensitive than virus isolation or immunohistochemistry in detecting BVDV in tissues.

CONTROL BY VACCINATION

Prior to the emergence of virulent BVDV 2 strains, most BVDV infections of nonbreeding animals were thought to be benign and emphasis was placed on the prevention of fetal infections. Thus, it was recommended that all breeding females be vaccinated prior to conception (Baker, 1987; Radostits and Littlejohns 1988). Other animals in the herd were

often not vaccinated. In the 1990s, with the emergence of virulent BVDV 2, the failure to vaccinate all animals in a herd proved to be disastrous, with losses of up to \$40,000 to \$100,000/herd when outbreaks due to these virulent viruses occurred (Carman et al., 1998). Fortunately, the vaccines at that time, which contained only BVDV 1, did provide a measure of protection against acute infections with BVDV 2 and outbreaks could be controlled (Carman et al., 1998).

Although immunization with some vaccines containing BVDV 1 alone were protective against acute BVD caused by BVDV 2 (Cortese et al., 1998, Carman et al., 1998), apparent vaccine breaks did occur (Ridpath et al., 1994) and incorporation of BVDV 2 in vaccines was considered a priority. In addition to the prevention of acute disease by BVDV 2, a continuing concern was the degree of fetal protection against BVDV provided by vaccines in general. With the emergence of BVDV 2, this concern escalated with reports that vaccines containing BVDV 1 appeared not to be protective against fetal infection with BVDV 2 (van Campen et al., 2000).

van Oirschot et al. (1999) reevaluated the results of fetal protection experiments that were conducted in the 1970s to 1990s by six different research groups. They concluded that no vaccine had shown full fetal protection and that protection varied from 33–86%. In only one study were none of the fetuses from the vaccinated cows infected after challenge; however, one fetus from six unvaccinated control cows also remained uninfected (Brownlie et al., 1995). In this type of experiment it is important that all fetuses in the control group become infected to demonstrate the ability of the challenge virus to infect the fetus under the defined experimental conditions. Thus, in the latter study the degree of protection conferred by the vaccine was calculated to be 86% because only five of six fetuses from the control group became infected rather than 100% of them.

CONTROL WITHOUT VACCINATION

In the 1990s, eradication programs for BVDV on a national level were implemented in Sweden, Norway, Finland, and Denmark (Bitsch and Ronshølt, 1995). These programs were conducted without vaccination. In Sweden, the national program began in 1993 as a voluntary program entirely financed by producers (Alenius et al., 1996). In Denmark, the BVDV eradication program was initiated by dairy farmers in 1994 followed by a government order to support the program in 1996 (Houe, 1996). Both the

Swedish and Danish programs involve classification of herds by their BVDV status, removal of persistently infected cattle from infected herds, monitoring herd status, and prevention of infection in BVDV-free herds. Since these countries do not vaccinate, antibody-positive animals act as indicators of infection. Thus, bulk milk testing for antibodies constitutes an important component of these programs and is used to classify and monitor herds as to their BVD status. In Sweden, bulk milk testing is done with an indirect ELISA and in Denmark with a blocking ELISA (Alenius et al., 1996; Houe, 1996). These programs significantly reduced the prevalence of BVDV-positive dairy herds after only a few years. For Sweden, the prevalence dropped from 51% in 1993 to 24% in 1995; for Norway, from 23% in 1993 to 14.4% in 1996; and for Denmark, from 39% in 1994 to 9% in 1999 (Alenius et al., 1997; Bitsch and Ronshølt, 1995; Bitsch et al., 2000; Waage et al., 1997). Finland, which began its program in 1994 with a very low prevalence (only 1%) in dairy herds, further reduced the prevalence to just 0.4% in 1997 (Nuotio et al., 1999).

Bitsch et al. (2000) reported that for the Danish program, legislation was needed in 1996 to ensure that no persistently infected cattle were allowed on common pastures. Movement of cattle to other herds or common pastures was allowed only after blood testing and certification that the animals were not persistently infected. At first, pregnant (non-persistently infected) cows were not controlled. Instead, all buyers of pregnant animals were advised to isolate and ensure a non-persistently infected status of their calves before introducing them into a new herd. Since not all farmers heeded these recommendations and herds became infected because of persistently infected calves resulting from the purchase of pregnant animals, legislation was modified to restrict the movement of all female cattle over 1 year of age, specifically that no such cattle could be moved from non-free herds to other herds or common pastures.

2000 TO THE PRESENT

RESPIRATORY DISEASE

Respiratory diseases have plagued the feedlot industry for many years and are collectively considered to be the most significant cause of mortality for feedlot calves. Several recent studies have linked BVDV with bovine respiratory disease in feedlot cattle and another study has identified BVDV strains that can cause primary respiratory disease experimentally.

Martin et al. (1999) determined the antibody titers

to BVDV, infectious bovine rhinotracheitis, parainfluenza-3, bovine respiratory syncytial virus, and two mycoplasma strains in 32 groups of calves prior to entry, and 4–5 weeks after their arrival, at the feedlot. They then looked for an association of these infectious agents (by increases in antibody titers) with the risk of respiratory disease. Of all the agents, BVDV had the most consistent association with elevated risk of respiratory disease and lower weight gains. In another study, Haines et al. (2001) examined 49 cases of antibiotic-unresponsive, chronic disease (most often respiratory disease and/or arthritis) in Canadian feedlot cattle. By immunohistochemistry, they found that *Mycoplasma bovis* and BVDV were the most common pathogens persisting in the tissues of these animals. BVDV was found in lung and/or joint tissues in 20 of the 49 (41%) cases.

Shahriar et al. (2002) reported on the prevalence of pathogens in cases of chronic, antibiotic-resistant pneumonia with or without concurrent polyarthritis occurring in feedlot cattle in western Canada. They examined retrospective (1995–1998) and current cases (1999) by immunochemistry of lung and heart tissue and found that *Mycoplasma bovis* was present in 44 of 48 cases of the retrospective group and 15 of 16 of current cases; whereas BVDV was present in 31 of 48 retrospective and 9 of 16 current cases. From four positive virus isolations in the current group, BVDV type 1b was isolated in two cases and BVDV 2 was isolated from the other two cases. These researchers suggested that a synergism between *Mycoplasma bovis* and BVDV might occur in this syndrome of pneumonia with concurrent arthritis.

Later, Fulton et al. (2002) examined the prevalence of BVDV in stocker calves with acute respiratory disease and reported that BVDV type 1b was the predominant type involved. These researchers also noted that vaccines in the U.S. primarily contained BVDV type 1a, that some vaccines also included BVDV 2, but that only one vaccine contained BVDV 1b. They suggested that, for effective vaccination against BVDV type 1b, there should be demonstrated efficacy of current BVDV type 1a vaccines against type 1b. Alternatively, new components of BVDV type 1b could be included in current BVDV type 1a vaccines.

Baule et al. (1997, 2001) identified two new subtypes of BVDV 1 isolated in southern Africa and designated these as type 1c and type 1d. Type 1d viruses were found to be predominantly associated with respiratory disease. Two cytopathic viruses of this type were inoculated in calves intranasally or

intravenously. All inoculated calves developed respiratory symptoms. One of the isolates produced mainly nasal discharge and fever in calves; however, the other isolate produced a more severe disease that included ocular discharge, nasal discharge, fever, coughing, abnormal breathing, and oral erosions. Transient diarrhea was observed in only 2 of 10 calves. There was a widespread distribution of virus in various tissues and organs of infected calves, including heart muscle, skin, bone marrow, and brain. However, lesions were mainly observed in the respiratory tract (focal catarrhal bronchopneumonia and atelectasis of the lung) and lymphoid tissues. In one calf, virus was still present in tissues 31 days after infection in the absence of viremia. Furthermore, two calves were still shedding cytopathic BVDV in nasal secretions at 21 and 31 days after infection when virus was no longer detectable in blood.

MOLECULAR ACTIONS OF CYTOPATHIC AND NONCYTOPATHIC BVDV

In recent years, the actions of cytopathic and noncytopathic BVDV on cells have been studied more intensively. For cytopathic strains of BVDV, it has been found that, like many other “lytic” viruses, they kill cells by triggering apoptosis (programmed cell death) rather than lysis and necrosis (Zhang et al., 1996; Adler et al., 1997; Hoff and Donis, 1997). Apoptosis is responsible for the elimination of cells in normal developmental processes but can be triggered by many stimuli and is characterized by condensation of chromatin, cell shrinkage, generation of apoptotic bodies, and fragmentation of chromosomal DNA with the generation of typical oligonucleosomal fragments. The mechanism(s) by which cytopathic BVDV triggers apoptosis is currently an active area of research. BVDV is an ideal model system for viral-induced apoptosis because both cytopathic and noncytopathic forms of the virus exist. In addition to the production of NS3, which is correlated with cytopathic effect, it has recently been discovered that cytopathic viruses accumulate much higher levels of viral RNA in cells than do noncytopathic viruses.

Vassilev and Donis (2000) used an infectious cDNA clone of the NADL strain to create, by deletion of the cellular insertion in the NS2 gene, an isogenic, noncytopathic virus mutant. To study viral RNA accumulation in cells, they utilized both cytopathic and noncytopathic (mutant) forms of the NADL strain and several naturally occurring cytopathic and noncytopathic viral pairs. At a multiplicity of infection of one, viral RNA accumulation was

6.5 to 23 times higher when cells were infected with the cytopathic virus than with the corresponding noncytopathic virus. Thus, in addition to generation of NS3, a second factor—increased viral RNA concentration in cells—may contribute to the ability of cytopathic BVDV to induce the death of cells.

Studies spanning several decades have shown that noncytopathic strains of BVDV do not induce type 1 (alpha/beta) interferons in cells (in vitro) and block the induction of interferons by other viruses (Diderholm and Dinter 1966; Nakamura et al., 1995; Adler et al., 1997). In contrast, cytopathic strains of BVDV do induce type 1 interferons in cells in vitro (Adler et al., 1997). Recently, Schweizer and Peterhans (2001) showed that noncytopathic BVDV inhibits the induction of apoptosis and interferons by a synthetic double-stranded RNA (poly IC). Subsequent work indicates that noncytopathic BVDV blocks an interferon regulatory factor (Baigent et al., 2002). These studies explain the basis of two somewhat obscure diagnostic tests for noncytopathic BVDV. The first, described in 1968, was the END method (enhancement/exaltation of Newcastle disease virus) (Inaba et al., 1968). Newcastle disease virus (NDV) induces, and is sensitive to, interferon. Co-infection of noncytopathic BVDV and NDV leads to an enhancement of the replication of NDV. In the END method, the presence of noncytopathic BVDV is thus shown by NDV enhancement due to the suppression of interferon by noncytopathic BVDV. The second diagnostic test was based on the suppression of an effect of poly IC (Maisonnave and Rossi, 1982). Cells were inoculated with noncytopathic BVDV, and then treated with poly IC, and subsequently inoculated with vesicular stomatitis virus (VSV). Cells infected with noncytopathic BVDV were not protected against cytopathic effect by VSV, whereas cells that were not infected with noncytopathic BVDV, were protected. It is now clear that the action of noncytopathic BVDV is via inhibition of interferon induction by poly IC.

The interference with interferon production in vitro by noncytopathic BVDV has been suggested as an enabling factor in the ability of these viruses to establish persistent infections in the early fetus (Schweizer and Peterhans, 2001). To determine whether interference occurs in vivo, Charleston et al. (2001) inoculated the amniotic fluid of approximately 60-day-old fetuses with noncytopathic or cytopathic BVDV. Whereas cytopathic BVDV induced interferon production in the fetus, noncytopathic BVDV failed to do so. In contrast, the dams of the fetuses inoculated with noncytopathic BVDV did

produce interferons, as did calves in a subsequent study (Charleston et al., 2002). Thus, there appears to be a marked difference in the interferon response between the early fetus and immune-competent animals upon infection with noncytopathic BVDV.

AN OLD VIRUS, ONCE LOST, TEACHES LESSONS

Of the old cytopathic BVDV strains—those that have been used in laboratories for many years—the NADL strain that was isolated from a case of MD in 1962 (Gutekunst, 1968) is still one of the most widely used in research and diagnostics and as a component in vaccines. As for other old cytopathic strains, the noncytopathic counterpart of the cytopathic NADL strain had been lost. However, recently, this virus has made an unexpected appearance; it was found, through in vivo studies, to be still residing in a stock of cytopathic NADL virus originally obtained from the American Type Culture Collection (Harding et al., 2002). Pregnant cows (at 90–105 days of gestation) were inoculated with virus originating from an infectious cDNA clone of NADL (i-VVNADL), or the parental NADL virus stock (termed NADL-A), and 3 or 6 weeks after inoculation the fetuses were harvested. Virus isolated from cows inoculated with iVVNADL was always cytopathic in biotype and no virus was isolated from fetuses from these cows. Surprisingly, viruses of both biotypes were initially isolated from cows inoculated with NADL-A, but after 8 days postinoculation only noncytopathic virus was recovered. Virus was isolated from 8 of 10 fetuses from dams inoculated with NADL-A. In each case, the virus was noncytopathic.

The nucleotide sequence of the NS2-3 and NS5A regions of the genome of the contaminating noncytopathic BVDV (termed NADL-1102) in the NADL-A stock was determined and the virus was found to lack the 270 base pair cellular insertion in the NS2 gene of NADL-A. Aside from the lack of the cellular insertion, which had been shown previously to be responsible for cytopathogenicity (Mendez et al., 1998), a greater than 99% homology exists between sequences of NADL-1102 and published NADL strain sequences. This level of homology indicates that NADL-1102 is the ancestral noncytopathic NADL virus from which the cytopathic NADL virus arose by genetic recombination. The authors of this report state that there had been no systematic attempts to eliminate noncytopathic BVDV from this stock by the repository personnel or the depositors (Gutekunst and Malmquist, 1963).

This study illustrates well, and confirms the findings of others, that noncytopathic BVDV, but not cytopathic BVDV, can infect the fetus and establish persistent infections (McClurkin et al., 1984; Brownlie et al., 1989). Prior to 1984, it was not appreciated that both BVDV biotypes could be isolated from cases of MD. Thus, cytopathic BVDV isolates may not have always been purified or adequately purified. For instance, plaque purification may not be adequate for purification of cytopathic BVDV without additional immunochemical or immunofluorescent staining to rule out the presence of noncytopathic BVDV. McClurkin et al. (1984), in their classic study on the production of immune-tolerant and persistently infected calves, produced persistent infections with noncytopathic BVDV but not with the cytopathic NADL strain. Apparently, their NADL stock virus was free of noncytopathic BVDV.

DIAGNOSIS

Within the past few years, several studies have examined the use of immunohistochemistry (IHC) on skin biopsies to detect BVDV infections, a technique that had been introduced earlier by Thür et al. (1996) for use on frozen specimens. The skin biopsy technique is now being used by some laboratories to screen herds for persistently infected cattle. Njaa et al. (2000) applied the technique on formalin-fixed, paraffin-embedded specimens and were able to detect positive staining in 41 of 42 skin samples from persistently infected calves. Sections of skin from all acutely infected animals were negative for staining when they were infected with 10^5 TCID₅₀ of BVDV, and only 40% of acutely infected animals showed positive skin biopsies when infected with a much higher dose (10^8 TCID₅₀) of BVDV. It was concluded that immunohistochemical staining of skin biopsies was an effective method for identifying persistently infected cattle.

Ridpath et al. (2002) used virus isolation as the standard to evaluate both the skin biopsy method and a serum PCR-probe test, another technique used for screening herds for persistently infected animals, for detection of acute BVDV infections. In this study, 16 animals were inoculated with 10^6 TCID₅₀ of BVDV 1 or BVDV 2. Whereas virus was isolated from all animals, only three (19%) animals were positive by the PCR-probe test and none was positive by immunohistochemical (IHC) staining of skin biopsies. These researchers concluded that the skin biopsy test would usually not confuse persistently infected animals with acutely infected animals. However, both methods (IHC and PCR-probe) were

considered unreliable for detecting acute outbreaks caused by BVDV.

Recently, the use of skin biopsies as diagnostic specimens for a BVDV antigen capture ELISA was evaluated (Brodersen et al., 2002). The assay, based on the detection of the E^{rms} protein, was reported to be equally sensitive to PCR, IHC, and virus isolation from leukocytes. The same capture ELISA had been previously validated for the detection of BVDV in serum for screening herds for persistently infected animals and for screening BVDV in sublots of fetal bovine serum for the production of the commercialized product (Plavsic and Prodafikas, 2001).

The use of pooled samples for diagnostic testing, a trend that began in the 1990s, has continued in recent years. Weinstock et al. (2001) developed a single tube PCR assay for detection of BVDV in pooled serum. The assay was compared with a microplate virus isolation method performed on individual sera. The PCR assay was sensitive enough to detect a single viremic serum sample in 100 pooled samples. Muñoz-Zanzi et al. (2000) examined least-cost strategies for using PCR/probe testing of pooled blood samples to identify persistently infected animals in herds. For a herd prevalence of 1%, the least-cost strategy for pooled sample testing was to test pools from 20 animals initially, and then pools of five blood samples for repooled testing. As herd prevalence increased beyond 3%, the competitive benefit of pooled testing diminished.

Based on earlier work of Houe (1992; 1994) and Houe et al. (1995), who reported that antibody levels can be used to predict the presence of persistently infected animals in a herd, Pillars and Grooms (2002) applied this technique to herds with unknown BVDV status. Specifically, they examined whether serological evaluation of five unvaccinated, randomly selected, 6- to 12-month-old heifers is a valid method for identifying herds that contain persistently infected cattle. A herd was classified as likely to contain persistently infected animals when at least three of the heifers had neutralizing antibody titers of $\geq 1:128$ to BVDV 1 or BVDV 2. Of the 14 herds examined, 6 contained persistently infected animals. The sensitivity and specificity of the serologic method for determining which herds had persistently infected animals was 66% and 100%, respectively. In one herd with a false negative result, by chance three of the heifers examined serologically were persistently infected animals and had titers of $<1:4$, whereas the other two animals had titers of $>1:4,096$. The authors noted that this herd would have been rightly classified had virus isola-

tion been used in addition to the serological testing. In the other herd with a false negative result, a single persistently infected calf of 3 months of age was missed. This animal was reported not to have had contact with the target population. The authors noted that the overall sensitivity of the serologic evaluation of five unvaccinated heifers for determining herds with persistently infected cattle could be improved by periodic retesting of herds that had negative results. The use and testing of sentinel animals in vaccinated herds has been recommended as an important element of a BVDV control program (Dubovi, 2002).

CONTROL BY VACCINATION

In recent years there have been two major trends in vaccine research and development: the inclusion of BVDV 2 strains in commercial vaccines and the evaluation of the efficacy of BVDV vaccines for protection against fetal infection. The latter, an assessment of efficacy for fetal protection, has not been a requirement for the licensing of vaccines in the U.S. and Canada. For licensing, the vaccines are required only to show protection against acute infections. However, in recent years, efforts by both individual researchers and vaccine companies have been intensified to evaluate BVDV vaccines for their efficacy in providing fetal protection. The ability to demonstrate and claim vaccine efficacy for fetal protection should give those vaccine companies a competitive advantage over others that cannot make such a claim for their products. This will be the driving force for new and better products in the absence of new regulations.

Recently, experiments involving immunization with BVDV-specific nucleic acids (DNA or RNA) have been performed. The first of these utilized a recombinant plasmid containing the BVDV E2 gene with a human cytomegalovirus promotor for DNA immunization of mice (Harpin et al., 1997). Neutralizing responses against BVDV 1 strains were generated, which persisted for 6 months after the last injection. In cattle, immunization with the plasmid produced both a neutralizing antibody response and a cellular immune response, but immunization was only partially protective when the cattle were inoculated with a challenge virus (Harpin et al., 1999).

Vassilev et al. (2001) used full-length, infectious BVDV RNA derived from a recombinant, infectious cDNA clone (NADL strain) to immunize cattle and sheep against BVDV. The RNA was coated onto gold microcarrier particles and delivered to the inoc-

ulation sites using a gene gun delivery system. Serumneutralizing antibody titers of $>2^{12}$ and $>2^7$ were generated in calves to the NADL strain of BVDV 1 and to a BVDV 2 strain, respectively.

Muñoz-Zanzi et al. (2002) developed models to predict the age of dairy calves when colostrum-derived BVDV antibodies would decline to a level that would no longer offer disease protection or interfere with vaccination. The ages at which 50% of the calves were predicted to have low antibody titers ($<1:16$) were, for two herds, 107–111 days for BVDV 1 and 75–81 days for BVDV 2. The ages at which 50% of the calves were predicted to be seronegative (titer $<1:8$) were, for the same herds, 139–143 days for BVDV 1 and 110–118 days for BVDV 2. These ages were considerably shorter than earlier estimates for the persistence of colostral antibodies (6–10 months). The differences in these estimates may be due, in part, to the large sample size (466 calves) used in this study or due to differences in protocols for administering colostrum.

ERADICATION

The eradication programs without vaccination that began in the 1990s in the Scandinavian countries have proven to be very successful in reducing the number of herds with BVDV infections. For instance, 52% of herds were estimated to be infected in Sweden in 1993, whereas in 2002, 93% of dairy and 88% of beef herds were considered BVDV-free (Lindberg, 2002). Eradication of BVDV appears to have been even more successful in Denmark where, in 2002, approximately 99% of dairy and 98% of beef herds were free of BVDV (Bitsch et al., 2002).

In Germany, an ongoing control program for eventual BVDV eradication is voluntary and partially subsidized by the state (Greiser-Wilke, 2002). Vaccination is practiced in Germany to reduce virus circulation and to minimize economic losses. A killed vaccine is used for basic immunization and an attenuated live virus vaccine is used to booster the immune response. Screening for removal of persistently infected animals is performed using an antigen capture ELISA. If all animals up to 3 years of age in a herd are negative, the herd is classified as “BVDV unsuspicious.”

In 2002, the Academy of Veterinary Consultants (AVC), presented a position statement on BVDV for eventual eradication of the virus in North America at a USDA-sponsored meeting (Grotelueschen, 2002). The AVC, established in 1970, is an association of veterinarians involved in beef cattle medicine, herd health programs, and consultation. Its members con-

sist of veterinarians from both the U.S. and Canada. The BVDV position statement reads:

The beef and dairy industries suffer enormous loss due to effects of bovine viral diarrhea virus (BVDV) infection. The highly mutable nature of BVDV and the emergence of highly virulent strains of BVDV contribute to limited success of present control programs. Also persistently infected cattle are the primary source of infection and effective testing procedures are available to identify those infected carriers. Therefore, it is the resolve of the Academy of Veterinary Consultants that the beef and dairy industries adopt measures to control and target eventual eradication of BVDV from North America.

The AVC position statement is likely only a first step toward a more comprehensive program against BVDV in North America, and the support of producer groups will be critical before such a program becomes a reality. The consensus of those present was that any eradication program in North America will likely include vaccination, and that vaccines that provide maximum fetal protection will be crucial to the success of this type of program.

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2

Risk Assessment

Hans Houe

INTRODUCTION

Many biological variables can be used to quantify the occurrence of bovine virus diarrhea virus (BVDV) infections, including clinical, pathological, virological, serological, and production measures. The importance of each measure depends on the purpose of the investigation. In surveillance and eradication programs, the laboratory diagnoses are important to document where the infection is present or was present recently; from an economic point of view it is important to combine virological and serological measures with information on clinical and production variables; and from an animal welfare point of view the emphasis would be on the severity of the clinical signs. However, it is not always possible to fully describe the occurrence of a disease for all purposes, simply because of the variations in the quality of the data.

For BVDV infections, good quality data are available from many parts of the world on the presence of persistently infected (PI) animals and antibody carriers in addition to epidemiological data on the occurrence of infection. However, when it comes to the disease (clinical signs and reduced production due to BVDV infections), the data are often based on selected cases, making it difficult to estimate the true occurrence of the disease in a population. Therefore, the calculation of the economic effect of infection is often based on the proportion of infected animals that are estimated to have suffered from the disease, which are hereafter included in economical models. Also the importance of risk factors is usually related to the occurrence of infection rather than that of the disease. This chapter outlines the occurrence of BVDV infections, the associated risk factors for infection and the effect of infections on disease and production including financial losses.

MEASURABLE AND QUANTIFIABLE EPIDEMIOLOGICAL VARIABLES

The relevant variables for describing the occurrence of BVDV infection include the detection of virus and/or antibodies in the animal as well as data related to disease and reduced production following infection. In addition, the occurrence of BVDV should be described in relation to risk factors that can be host-, agent-, or environment-related. This chapter emphasizes risk factors for determining the occurrence and distribution of BVDV. An epidemiological framework for the necessary data for describing BVDV occurrence is given in Figure 2.1. Here the variables are divided into risk factors on the one hand and measurable output variables on the other. The list of epidemiological data in Figure 2.1 is not exhaustive, but the boxes in the figure refer to the following subchapters where more detailed information is given.

There are two important mathematical measures of disease occurrence (or disease frequency): prevalence and incidence. The *prevalence* is a measure of what is present here and now, whereas *incidence* measures the occurrence of new cases over time. Both measures are useful in describing BVDV infections. The occurrence of long-lasting antibodies (often lifelong) and the lifelong presence of virus in PI animals make prevalence studies more suitable for BVDV infections than is the case for many other infections. Prevalence and incidence can be applied both to individual animals as well as herds. However, the definition of a herd infection may often be more uncertain and more complicated than at the individual animal level.

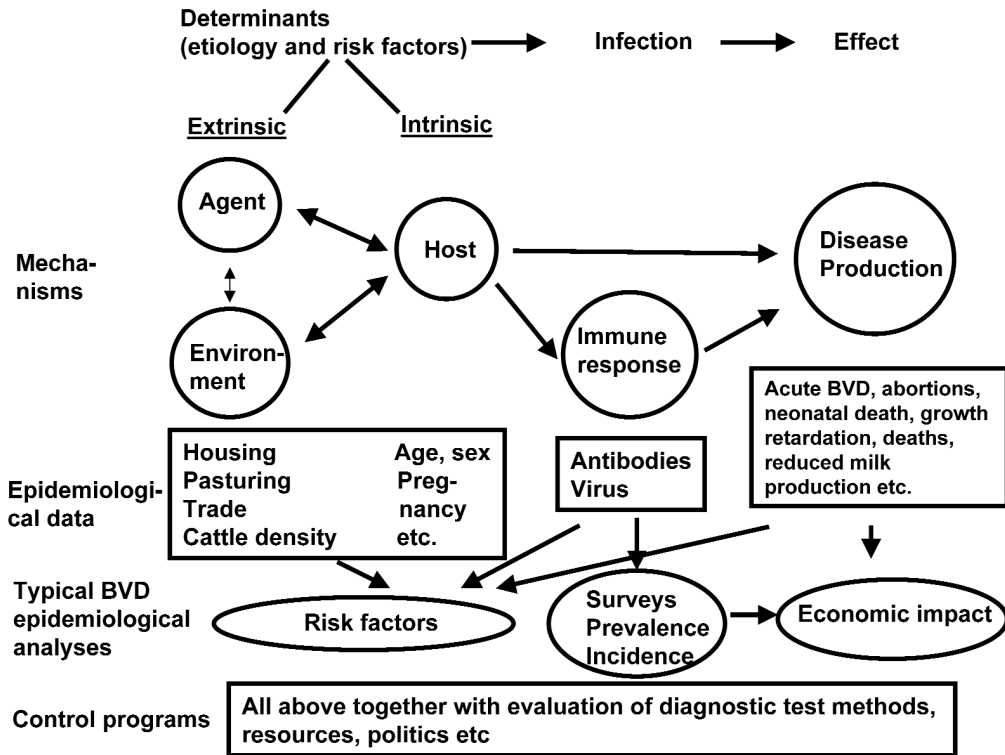


Figure 2.1. An epidemiological framework for the necessary data for describing BVDV occurrence, risk factors, and effect.

PREVALENCE OF BVDV INFECTIONS

The prevalence of a disease or infection (or any other condition) is defined as the proportion of the population with the disease at a given point in time:

$$p = \frac{\text{Number of diseased or infected animals at time } t}{\text{Number of animals in the sample at time } t}$$

The prevalence can be interpreted as the probability that a randomly selected animal has the disease at time t . Thus, the prevalence of BVDV antibody carriers is the proportion of animals having antibodies, no matter when they were acquired. The advantage of a prevalence study is that animals need to be sampled only once. The disadvantage is that it is not known when the antibodies were acquired, or how. However, for PI animals we know that they were infected in the first trimester of fetal life (although viremic animals need to be retested after 2–3 weeks, it is still considered a prevalence study), and hence prevalence studies on the occurrence of PI animals are very useful.

Diagnostic methods for the detection of BVDV

and its antibodies are covered in detail in Chapter 12. In the context of describing occurrence, it is important to evaluate prevalence studies in relation to the sensitivity and specificity of the diagnostic tests. If, for example, a serologic test has revealed a prevalence of 45% antibody carriers (apparent prevalence, AP) and the test is known to have sensitivity (Se) of 97% and a specificity (Sp) of 88%, the true prevalence (TP) can be calculated from this formula:

$$TP = \frac{AP + Sp - 1}{Sp + Se - 1} = \frac{0.45 + 0.88 - 1}{0.88 + 0.97 - 1} = 0.39$$

where AP denotes apparent prevalence (the prevalence of test-positive animals). This means that the true prevalence is 39%. These calculations are made with the assumption that all serologically positive animals arose via natural infection. Antibodies titers that are due to vaccination will throw off these calculations.

For the detection of both virus and antibodies in individual animals, the sensitivities and specificities are usually very high and often close to 100%. For detection of antibodies, the Se and Sp of different

ELISA tests as compared to serum neutralization test have often been higher than 90% (Cho et al., 1991: $Se = 97.6\%$ and $Sp = 100\%$; Rønsholt et al., 1997: $Se = 96.5\%$ and $Sp = 97.5\%$; Canal et al., 1998: $Se = 97.5\%$ and $Sp = 99.2\%$; Kramps et al., 1999: $Se = 98\%$ and $Sp = 99\%$). Comparison of BVDV antigen ELISA with conventional tissue culture isolation technique has also shown high sensitivity and specificity (Rønsholt et al., 1997: $Se = 97.9\%$ and $Sp = 99.5\%$).

Many studies give estimates on the prevalence of infected herds. Here it is important to know that the definition of an infected herd can vary substantially and that the sensitivity and specificity can show higher variation and will often be known with a lower certainty than at the individual animal level. Correct classification of herds into being truly infected or noninfected has important implications for observational studies on epidemiological aspects—e.g., prevalence, incidence, identification of risk factors, and quantification of the effect of BVDV infection on disease and production.

The term *herd test* or *herd diagnosis* is based on testing samples from more than one animal. The sample can consist of separate samples from individual animals (e.g., blood samples) or it can be a pooled sample from several animals (e.g., pooled blood samples or a bulk tank milk sample). If the samples consist of individual animals with a sample size of n , and the herd is defined as positive if there is at least one positive animal in the herd, the herd sensitivity (HSe) and herd specificity (HSp) can be calculated from the prevalence of test-positive animals (AP) and from the specificity at animal level (Sp) as follows:

$$HSe = 1 - (1 - AP)^n$$

and

$$HSp = Sp^n$$

From the formulas it can be seen that increasing the sample size will result in increased herd sensitivity but decreased herd specificity. Furthermore, a high prevalence would increase herd sensitivity but would not affect herd specificity. These formulas are true only when definition of a herd is based on one or more animals being positive (i.e., the critical number is 1). Sometimes, a herd is defined positive only if a higher number of animals are positive and in these cases other approaches are needed (Martin et al., 1992). In many published epidemiological studies the herd prevalence is given based on at least

one animal being positive for either antibody or virus.

The herd diagnoses for the presence of PI animals are often based indirectly on an interpretation of serological test results of screening young stock, from antibody reaction in bulk tank milk or combinations hereof. The true status should then preferably be based on examination of all individual animals. In 26 herds where blood testing of all animals was used as the gold standard, the HSe and HSp of using a small screening sample of young stock was calculated as 0.93 and 1.0, respectively (Houe, 1999). Others have reported HSe and HSp of 0.66 and 1.0, respectively, using serologic results from unvaccinated heifers among 14 dairy herds (Pillars et al., 2002). The reason for a low HSe can be that the PI animals are young (Houe, 1992b; Houe, 1999; Pillars et al., 2002) or it has even occurred that by chance the PI animals were selected for serologic testing giving negative results (Pillars et al., 2002). Therefore repeating the serologic testing of young stock a few months later and also having them tested for virus will increase HSe significantly.

The antibody level in bulk tank milk has shown good correlation with the prevalence of antibody carriers in a herd (Niskanen, 1993; Houe, 1994; Beaudeau et al., 2001). Using a screening sample of young stock as a gold standard, a blocking ELISA measuring antibody levels in bulk tank milk was evaluated. Among 352 dairy herds the bulk tank milk antibody test had a sensitivity of 1 and a specificity of 0.62 at a blocking percentage (cutoff) of 50% (Houe, 1999). Furthermore, at an estimated herd prevalence of 26%, the positive and negative predictive values were 0.48 and 1.00, respectively. This means that the bulk tank milk test is very good at detecting herds with true PI animals. On the other hand, many herds without PI animals may also have high antibody levels (e.g., due to a PI animal moved from the herd before the test), and therefore the test result is false positive. In some areas, there may be poor predictive values of antibody detection among a few young stock or antibody level in bulk milk for herd diagnosis because of high prevalence of infection and large variation of antibody-positive animals in herds without PI animals (Zimmer et al., 2002).

Many studies use an increase in bulk tank milk antibody level as an indication of a recent infection in the herd (Niskanen et al., 1995). Also PCR on bulk milk has been used to directly detect virus excretion in the herd (Drew et al., 1999). Unfortunately, there are no international standards for clas-

sification of herd infection status, and one should be careful when comparing studies that use different definitions for an infected herd.

EPIDEMIOLOGICAL STUDIES FOR ESTIMATION OF PREVALENCE

Due to the development of faster and cheaper diagnostic methods the number of prevalence studies has increased significantly in recent years. When comparing different studies one should be aware of differences in study design, study period, sampling frame, sampling method, sampling units, sample size, and the exact test variable being measured.

Tables 2.1–2.3 summarize a number of prevalence studies including some keyword information on sampling frame and sampling methods. These studies were selected to represent a broad variety of different geographical regions. Another criterion for selection was that the studies contained sufficient information on study design and sampling strategies to be judged fairly representative for the study area. Attempts were made to include both older and newer prevalence studies. Studies in countries with national control and eradication programs are from before or at the beginning of the eradication programs.

The sampling frame in the tables specifies whether samples have been taken from certain regions in the country and whether there is information on the type of enterprise (dairy herds, beef herds, breeding herds, or artificial insemination centers). The exact sampling methods are often rather complicated and for details the reader is referred to the cited publications. The tables include information on the major criteria for sampling such as the randomness and representativeness of the samples and whether only certain age groups are sampled. The sampling has often been done by initially selecting herds (primary sampling units) followed by sampling of individual animals (secondary sampling units). But there are also examples where animals are the primary sampling unit (e.g., sampling from abattoirs) or where herds are sampled without further sampling of animals (e.g., testing for antibody level in bulk milk, as shown in Table 2.3).

The variables behind the prevalence measures when testing individual animals are either antibodies (Table 2.1) or virus (Table 2.2). The same study can, therefore, be represented in both tables if animals are tested for both antibodies and virus. The prevalence can be given both at the animal level as well as at the herd level (here defined as the prevalence of herds with any antibody or virus-positive animal). In addition, many studies aim directly at

obtaining a herd infection status by serological testing of a few young stock, testing the bulk tank milk for antibody or virus, or by various combinations of these methods. These studies provide prevalence estimates of herds with any antibody carriers or presence of any PI animals (or rather herds being suspected of having PI animals). As these studies are often based on different interpretations of antibody level in bulk tank milk, they are presented separately in Table 2.3 (some keywords of the interpretation from the studies are given in the table). Altogether, the following six prevalence measures are presented in Tables 2.1–2.3:

1. Prevalence of antibody-positive animals
2. Prevalence of virus-positive animals
3. Prevalence of herds with at least one antibody carrier
4. Prevalence of herds with at least one virus-positive animal
5. Prevalence of herds with antibody-positive animals as detected from screening samples (spot tests) and bulk milk
6. Prevalence of herds with high probability of virus-positive animals as detected from screening samples (spot tests), antibody level in bulk milk, or PCR on bulk milk

One of the difficulties of interpreting serological results is the very different use of vaccines. When there were clear indications of the use of vaccine, either in the country or in the study herds, this information is provided in the tables. One should also be careful interpreting the prevalence of infected herds if not all animals in the herd are tested.

Due to the various sampling strategies and interpretation of test results, formal statistical analyses of Tables 2.1–2.3, estimation of confidence intervals (CI) of the true occurrence, and testing for differences between different areas are not straightforward. Instead, many different studies are outlined in the tables in order to provide the reader with a spectrum of results from different areas, emphasizing some selected trends. The reader should be aware of the sampling methods when comparing prevalence. If, for example, only younger animals were sampled for the detection of PI animals, the prevalence would be higher than if sampling was done among all animals (simply because PI animals die faster than other animals).

Considering the prevalence of antibody carriers at the animal level, there seems to be a continuum of prevalence levels from a minimum of 12% to a maximum of 89% (Table 2.1). However, if all the pre-

Table 2.1. Epidemiological studies for estimation of prevalence of animals with antibodies against BVD

Country/ Region	Study Period	Sampling Method				Sample Size		Prevalence		Reference
		Sampling Frame	Herds	Animals	Herds	Animals	Herd Level (%)	Animal Level (%)		
<i>Europe</i> Belgium	...	Southern Belgium Belgian White Blue and Friesian Holstein	42.5% of herds had prior diagnosis or were suspicious	All animals in herd	61	9685	100	66	Some vaccination (not considered important)	Schreiber et al., 1999
Denmark	1988	Jutland in Denmark Dairy herds	Representative NPE	All animals in herd	19	2570	100	64	No vaccination	Houe and Mey- ling, 1991
Netherlands	Random among ani- mals in BHV1 ^a diagnostics and field trial	>100	1798	—	65	...	Kramps et al., 1999
Norway	1984–1986	Wide geographic representation Dairy herds	Geographic repre- sentative, NPE	Random >2 years	187	1133	28	19	No vaccination	Løken et al., 1991
Poland	...	Bulls at artificial insemination centers	—	>6 months old	—	175	—	86	...	Polak and Zmudzinski, 1999
Scotland	1992–1993	South West Scotland Breeding bulls on dairy, beef of mixed farms (5 bulls from dealers)	...	Random	78	109	—	78	...	McGowan and Murray, 1999
Slovenia	1996	5 regions Breeding herds	...	All animals in herd	274	6892	...	17	...	Grom et al., 1999
Spain	1997	Asturias region Dairy herds	Random/stratified NPE	20 herds: all animals 8 herds: Random, >1-year-old	28	529	86	21	No vaccination	Mainer-Jaime et al., 2001
Sweden	...	11 counties in dif- ferent parts of Sweden	NPE	Breeding heifers	114	711	—	41	No vaccination	Alenius et al., 1986
Sweden	1987	County of Koppar- berg Dairy herds	Random	All lactating cows	15	413	73	46	No vaccination	Niskanen et al., 1991
										<i>(continued)</i>

(continued)

Table 2.1. Epidemiological studies for estimation of prevalence of animals with antibodies against BVDV (*continued*)

Country/ Region	Study Period	Sampling Method				Sample Size		Prevalence		Reference
		Sampling Frame	Herds	Animals	Herds	Animals	Herd Level (%)	Animal Level (%)		
Switzerland	1994–1995	Canton of St. Gallen Dairy herds	Random	Cows and heifers (All)	95	2892	100	84	...	Braun et al., 1997
Switzerland	1995	Canton of St. Gallen, 7 Alpine pastures Swiss Braunvieh cattle	Invited by cantonal veterinary officer	Animals prior to pasture, 98% were replacement cattle NPE	149	990	—	63	...	Braun et al., 1998
Switzerland	1993–1994	Dairy herds	Random (at least 5 cows)	All cows	113	1635	99	72	...	Stärk et al., 1997
United Kingdom	1974–1975	England and Wales	3 herds in each county	12 per herd repre- senting a range of ages	133	1593	—	62	...	Harkness et al., 1978
United Kingdom	1985–1986	England and Wales	—	Submissions of more than 10 samples to Cen- tral Veterinary Laboratory	—	18,759	—	65	...	Edwards et al., 1987
<i>North, Central and South America</i>										
Canada	...	Saskatchewan and Alberta Several breeds Especially beef	Several sources: Brucellosis certification, bull test station, research station and others	295	1745	(67) ^b	41	Some vaccination. One-third of vaccinated had antibodies	Durham and Hassard, 1990	
Canada	1990–1991	Northwestern Quebec, beef herds (cow calf)	Random >25 breeding females	Systematic random among pregnant. 12–25 cows per herd	15	311	93	53	Only nonvacc. within 1 year included here	Ganaba et al., 1995
Canada	...	3 Maritime provinces: New Brunswick, Nova Scotia, Prince Edward Island Dairy herds	Stratified, two stage random sample	5 cows or 5 heifers >6 months old	89	445	(66)	38	Tested animals not vaccinated	VanLeeuwen et al., 2001

Table 2.1. Epidemiological studies for estimation of prevalence of animals with antibodies against BVDV (*continued*)

Country/ Region	Study Period	Sampling Method				Sample Size		Prevalence		Reference	
		Sampling Frame	Herds	Animals	Herds	Animals	Herd Level (%)	Animal Level (%)	Vaccination		
United States	1976	Louisiana	Research herds	>2 years old breeding cattle	5	444	100	68	No vaccination	Fulton and Seger, 1982	
United States	...	Midwest and western U.S. Beef and dairy herds	Appr. Half of the herds had past evidence of BVDV	Between 20 and 312 per herd Both calves and adults	66	3157	...	89	...	Bolin et al., 1985	
United States	1993	Michigan, 2 counties	5 herds ^c 2 herds ^d	All animals in herd	5 ^c 2 ^d	794 ^c 428 ^d	80 ^c 100 ^d	29 ^c 76 ^d	Vaccination used in 4 herds	Houe et al., 1995b	
United States	...	Dairy herds	2 herds ^e		2 ^e	372 ^e	100 ^e	91 ^e			
United States	...	17 states Beef cow calf operations	Representative of the national beef herd	Owner chose age group	256	3894	91 all 95 V ^f	69 all 78 V	Vaccination reported in 137 herds	Paisley et al., 1996	
Mexico	...	Dairy cattle: 2 states Beef cattle: 11 states	...	Random	...	132 (dairy) 771 (beef)	86 NV ^f —	57 NV 71 63	...	Suzan et al., 1983	
Brazil (Argentina)	...	State of Rio Grande ¶ 1 farm in Argentina	...	Adults, Abattoirs (mostly) Between 2 and 47 from each herd	20	430	90	56	...	Canal et al., 1998	
Chile	...	Southern part of Chile Beef, dairy and mixed breed	Large herds Stratified	Cows and bulls Random	40	948 cows 116 bulls	100	74 81	No vaccination assumed	Reinhardt et al., 1990	
Venezuela	1997	5 districts of Apure State Beef cattle	Multistage, random	Random 5 cows per herd	123	615	—	36	...	Obando et al., 1999	
<i>Africa, Asia and New Zealand</i>											
Tanzania	1985	18 regions	“Stock at Animal Diseases Research Institute, Dar-es-Salaam” NPE Age not recorded			...	419	—	12	No vaccination	Msolla et al., 1988
<i>(continued)</i>											

Table 2.1. Epidemiological studies for estimation of prevalence of animals with antibodies against BVDV (*continued*)

Country/ Region	Study Period	Sampling Frame	Sampling Method			Sample Size			Prevalence		Reference
			Herd	Animals	Herds	Animals	Herds	Animals	Herd Level (%)	Animal Level (%)	
Tanzania	1985–1986	5 regions, 11 different districts, Northern Tanzania	“Under the Auspices of the Rinderpest campaign”	NPE	...	938	34	...	Hyera et al., 1991
India	1996–1997	16 states									
			National serum bank		...	327			15	...	Sudharshana et al., 1999
New Zealand	...	South Island, Otago district	Random	Two abattoirs	—	64	—	—	66	...	Robinson, 1971
New Zealand	1993	North and South Island	14 abattoirs		70	140	(78) [§]		63	...	Pérez et al., 1994
		Beef cattle									

General legends and abbreviations in tables:

— Information not applicable.

... Information not available (the reader can of course add information on the use of vaccine from other sources).

NPE: No past evidence, meaning that herds were not selected based on past evidence of infection (unknown BVD status).

AI: Artificial insemination centers.

V: Vaccinated; NV: Not vaccinated.

^aBHV: Bovine herpes virus.

^bSample size varied considerably between farms.

^cHerds without use of vaccination and without PI animals.

^dHerds with use of killed vaccine; no PI animals present.

^eHerds with use of killed vaccine and presence of PI animals.

^fV: Prevalence among vaccinated; NV: Prevalence among nonvaccinated (herds were classified according to reported use of any BVDV vaccine during the previous 12 months).

[§]Note that there were only two sampled animals per farm.

Table 2.2. Epidemiological studies for estimation of prevalence of virus-positive and persistently infected animals

Country/ Region	Study Period	Sampling Method				Sample Size		Prevalence		Vaccination	Reference
		Sampling Frame	Herds	Animals	Herds	Animals	Herd Level (%)	Animal Level ^a (%)			
<i>Europe</i>											
Belgium	...	Southern Belgium. Belgian White Blue and Friesian Holstein	42.5% of herds had prior diagnosis or were suspicious	All animals in herd	61	9685	44	0.75 (PI)	Some vaccina- tion (not considered important)	Schreiber et al., 1999	
Denmark	1988	Jutland in Denmark Dairy herds	Representative, NPE	All animals in herd	19	2570	53	1.4/1.1	No vaccination	Houe and Mey- ling, 1991	
Germany	...	Northern Germany. Breeding animals	Exporting herds	Pregnant NPE	>1000	2317	—	0.9 (viraemic)	...	Liess et al., 1987	
Germany	1993–1994	Lower Saxony	NPE	Up to 3 years	329	20,253	45	2.1 (PI)	Some vaccina- tion	Frey et al., 1996	
Poland	...	Bulls at artificial insemination centers	—	>6 months old	—	219	—	0.9 (PI)	...	Polak and Zmud- zinski, 1999	
Sweden	...	11 counties in dif- ferent parts of Sweden	NPE	Breeding heifers	114	711	—	1.7/1.3	No vaccination	Alenius et al., 1986	
Switzerland	1995	Canton of St. Gallen, 7 Alpine pastures Swiss Braunvieh cattle	Invited by cantonal veterinary officer	Animals prior to pasture, 98% were replacement cattle NPE	149	990	—	0.9	...	Braun et al., 1998	
United Kingdom	1980–1985	Dairy herds ...	—	Beef calves 2–4 months old Cows 2–3 years old Gnotobiotic calves NPE	—	924	—	0.8/0.4	...	Howard et al., 1986	
United Kingdom	1986	England and Wales	—	Submissions of more than 10 samples to Cen- tral Veterinary Laboratory	—	3151	—	1.8 (viraemic)	...	Edwards et al., 1987	
										(continued)	

(continued)

Table 2.2. Epidemiological studies for estimation of prevalence of virus-positive and persistently infected animals (*continued*)

Country/ Region	Study Period	Sampling Frame	Sampling Method			Sample Size		Prevalence			Reference
			Herds	Animals	Herds	Animals	Herds	Level (%)	Animal Level ^a (%)	Vaccination	
North America											
Canada	1991	Western Canada feedlot calves	1 feedlot, but ani- mals were arriv- ing from many places	Systematic random, every 5th calf at arrival	—	1029	—	0.1	Taylor et al., 1995
Canada and U.S.	...	Northeastern and western states (2 AI centers throughout U.S. and Canada (2 AI centers)	—	Bulls at AI isolation prior to progeny testing. Mostly 3–12 months old	—	1532	—	0.4	Howard et al., 1990
United States	...	Midwest and western U.S. Beef and dairy herds	Appr. half of the herds had past evidence of BVDV ^b	Between 20 and 312 per herd Both calves and adults	66	3157	9.1	1.9/1.7	Bolin et al., 1985
United States	1993	Michigan, 2 counties Dairy herds	Random from DHIA list	All animals in herd	20	5481	15	0.13/0.11	Vaccination used in 15 herds		Houe et al., 1995b
United States	...	17 states Beef cow calf operations	Representative of the national beef herd	Owner chose age group. Only Ab- neg tested for virus	256	1201 Ab neg. out of 3894		0.67 of Ab negatives total	Vaccination re- ported in 137 herds		Paisley et al., 1996
United States	1996	Alabama, Nebraska, Nevada, North Dakota, and Ohio Beef herds	A. Random, 76 herds, min 20 and max 500 breed- ing females B. Suspected, 52 herds	Calves <4 months old (most >4 months old)	Total: 128 A: 76 B: 52	18,931	Total: 10.2 A: 4 B: 19	Total: 0.3/0.17	92% of positive and 82% of negative herds used vaccina- tion		Wittum et al., 2001

^aIf two percentages are given, the first number is prevalence of animals being viraemic and the second number is animals proven to be PI.

^bAmong 6 herds where virus was isolated only one herd had been selected based on past evidence.

Table 2.3. Epidemiological studies for estimation of herd level prevalence based on screening samples and bulk milk samples

All Countries

Country/ Region	Study Period	Sampling Frame	Sampling Method	Sample Size (Herds)	Sample	Herd Prevalence, Antibody Carriers (%) ^a	Herd Prevalence, PI Animals or Suspect of Having PI Animals (%) ^a	Reference
Austria	1996–1998	Nieder-österreich All breeding herds	Stepwise: A: milk, B: spot test and C: all animals NPE	A: 5,024 B: 512 C: 154	Milk Spot test All animals	...	1.0 (detection of PI animals)	Rossmannith and Deinhofer, 1998
Denmark	1994	Dairy herds	All herds	16,113	Bulk milk	...	39 (suspected to have PI)	Bitsch and Rønsholt, 1995
Estonia	1993–1995 1997–1998 1999–2000	Dairy herds with >=20 cows	Representative random sample	328 363 351	Bulk milk and/or young stock test	...	46 16 18 (suspected to have PI)	Viltrop et al., 2002
Finland	1993	Dairy herds	All herds (>98%)	34,115	Bulk milk	1	...	Nuotio et al., 1999
England	1996	Dairy herds	Systematic random	1070	Bulk milk	95 (OD >=0.135)	66 (OD >=0.9)	Paton et al., 1998
and Wales		>40 cows						
Northern	1999	Dairy herds	From the largest milk processor	929	Bulk milk	99 (OD >0.04)	50 (OD >=0.55)	Graham et al., 2001
Ireland								
Norway	1993	Dairy herds	All herds	26,430	Bulk milk	37 (OD >=0.05)	7.1 (OD >=0.55)	Waage et al., 1997
Peru	1998	Mantaro Valley	Proportional geographical distribution	60	Bulk milk	96 (OD >=0.05)	27 (OD >=0.55)	Ståhl et al., 2002
Sweden	1993	Dairy herds	Majority of dairy herds	14,463	Bulk milk	...	51 (OD >0.55)	Alenius et al., 1997
United	3-year period	Most herds: New York, Pennsylvania, and Michigan Remaining from 11 other states	...	97	Bulk milk PCR and virus isolation	—	12 (most likely PI animals among lactating animals)	Renshaw et al., 2000

^a Note that the antibody detection methods vary between countries as do the cutoffs when a herd is considered to have antibody carriers or PI animals. Prevalences are therefore just indicative of the level and not directly comparable between countries.

valence studies are ranked according to prevalence levels there is a tendency to different plateaus. Especially, a large number of studies have revealed prevalence from 60–70% of antibody carriers. At the low scale there are almost as many studies revealing prevalence in the range of 10–20% as there are from 20–60%.

Turning to the prevalence of virus-positive and PI animals it is evident that many studies find prevalence from 0.75 to around 2%. However, another group of studies has revealed prevalence in the range of 0.1–0.2% (Table 2.2). This is also reflected in the prevalence at the herd level where some studies have found 40–50% of the herds to host PI animals (Table 2.2) or being suspicious of hosting such animals (Table 2.3) whereas other studies have found only 10–15% of the herds to host (or being suspicious of hosting) PI animals (Tables 2.2 and 2.3). This could lead one to speculate that areas can be divided into high-and low-prevalence areas.

Looking at the regional differences, many European countries were found to have high prevalence. However, generalizations are not possible because some countries (e.g., Norway, Finland, and Austria) show low prevalence. It is noteworthy that prevalence studies in North America have revealed a very low prevalence of PI animals although the prevalence of antibody carriers in nonvaccinated herds in some studies has been relatively high. The reason for these differences can only be speculated upon. Variation in vaccination programs and management styles may result in differences. It can also be speculated that the mortality among PI animals may vary or that the outcome of fetal infection is different (i.e., the ratio of abortion versus birth of PI animals may vary). Furthermore, it would be reasonable to assume that some of the differences are due to variations in demographic circumstances (see later section, “Risk Factors for Occurrence of BVDV Infections”). In U.S. and Canadian studies it is also characteristic that PI animals can occur in high numbers in some herds.

Follow-up studies in some areas have shown that the infection can stay endemic in an area but with a pronounced change in infection status among the underlying herds (Houe et al., 1997). A study in Estonia showed a large decrease in herds suspected to have PI animals from 1993 to 2000 (Viltrop et al., 2002). The change was believed to be due to decreased trade of breeding animals. Further, it was proposed that the reduction of BVDV in these herds was facilitated by the facts that most large herds were closed and the density of cattle farms was low.

In Sweden, self-clearance has been fostered by controlling introduction of new animals (Lindberg and Alenius, 1999). However, without such specific interventions, BVDV infections will usually maintain themselves at high levels. In the Nordic countries, eradication programs were initiated in the beginning of the 1990s. In these countries the prevalence of herds with PI animals has been significantly reduced and is now approaching zero (Alenius et al., 1997; Bitsch et al., 2000; Valle et al., 2000a; Houe, 2001).

EPIDEMIOLOGICAL STUDIES ON OCCURRENCE OF DIFFERENT GENOTYPES

The variation of BVDV strains is described in detail in Chapter 3. On most occasions, the variation in BVDV is described at a qualitative level, but quantification of the strains in different regions and their relationship to risk factors is largely unknown. Among 96 field isolates collected in Germany between 1992 and 1996, 11 (11%) were identified as BVDV genotype 2 (Wolfmeyer et al., 1997). Another study of 61 field isolates collected between 1960 and 2000 in Northern Germany identified 2 isolates as genotype 2 (Tajima et al., 2001). It was also indicated that the virus population had been relatively stable over time. Among 28 field isolates from Belgium, only 1 belonged to genotype 1a and the remaining could be divided into genotypes 1b and 2 (Couvreur et al., 2002). Other isolations of BVDV genotype 2 in Europe include findings from Slovakia (Vilcek et al., 2002) and United Kingdom where the first definitive genotype 2 isolate was obtained in 2002 (Drew et al., 2002).

In the U.S., several strains gathered over the last many years have belonged to genotypes 1a, 1b, and 2 (Ridpath et al., 2000). Among a sample of 203 BVDV isolates from lots of pooled fetal bovine serum, 51 (25.1%) were identified as genotype 1a, 64 (31.5%) as genotype 1b, 65 (32%) as genotype 2, and 23 (11%) as a mixture of isolates (Bolin and Ridpath, 1998). Of the 105 BVDV isolates from diagnostic laboratory accessions, 61% belonged to genotype 1 and 39% to genotype 2 (Fulton et al., 2000). In South America, analysis of 17 Brazilian isolates identified 13 isolates (76%) as genotype 1 (4 as 1a and 9 as 1b) and 4 isolates (24%) as genotype 2 (Flores et al., 2000). In summary, BVDV genotype 2 seems much more prevalent in North America than in Europe. There are little or no data on prevalence in the African or Asian continents and only very limited data from South America thus far.

INCIDENCE OF BVDV INFECTIONS

The incidence of an event (disease, infection, or any other condition) is a measure of new cases within a given time period. The incidence is usually calculated relative to the population at risk. Two often-used measures are the incidence risk and the incidence rate. The incidence risk (I_{risk}) can be defined as follows (Toft et al., 2004):

$$I_{risk} = \frac{\text{Number of new cases in the study period}}{\text{Number of animals at risk at the start of the study period}}$$

The incidence risk can be interpreted as the probability that a susceptible animal (i.e., animal at risk) will experience the event of interest in a given time period. Thus the incidence risk can be interpreted at the individual animal level. If the incidence risk has been determined for a time period, the incidence risk for n time periods of the same length, where we assume that the incidence risk is constant throughout all individual periods, can be determined as follows:

$$I_{risk} = 1 - (1 - I_{risk(p)})^n$$

The incidence rate (I_{rate}) on the other hand is a measure of the speed (rate) at which healthy animals becomes diseased or infected. It is expressed per time unit (animal time at risk) and can mathematically be defined as:

$$I_{rate} = \frac{\text{Number of new cases in the study period}}{\text{Animal time at risk}}$$

The animal time at risk is the sum of the time at risk for each animal until it either becomes sick, is withdrawn from the study or the study ends. The I_{rate} is suitable for studies in dynamic populations where animals enter and leave the study during the study period, because it is a measure per animal time and not per animal, as is the case for the I_{risk} . If animals enter and leave the study population uniformly throughout the study period (i.e., on average they enter and leave in the middle of the study period) the time at risk can be estimated as follows:

$$\text{Time at risk} = \frac{\text{Number of animals at risk at start} + \text{Number of animals at risk at end}}{2} * \text{time period.}$$

Under the assumption that the incidence risk or incidence rate is constant during the study period,

the following relationship exists between the two measures:

$$I_{risk} = 1 - e^{-I_{rate} * t}$$

EPIDEMIOLOGICAL STUDIES FOR ESTIMATING INCIDENCE

There are few studies with direct determination of incidence of infection based on repeat sampling. It is, therefore, relevant to determine the incidence indirectly from prevalence measures (see next section, "Prevalence and Incidence"). The incidence of infection within herds or group of animals will depend on whether only transiently infected animals are present or both PI animals and transiently infected animals are present. The incidence of infection when only transiently infected animals are present varies from case to case. In a dairy herd where the last PI animal was removed in April 1987, 7 of 41 animals born in 1988 and 7 of 54 animals born in 1989 had seroconverted by December 1990 (Barber and Nettleton, 1993). As we do not know the birth dates of calves, the exact incidence rate cannot be calculated. However, assuming an average time at risk of 2 years for each animal, the total time at risk will be 95 animals of 2 years—i.e., a total of 190 animal years at risk. A total of 14 seroconversions in 190 animal years at risk gives an incidence rate of $14/190 = 0.073$ cases of seroconversions per animal year corresponding to an annual incidence risk of 0.067 (6.7%) using the shown formula for conversion between incidence risk and incidence rate.

In a long-term epidemiological study in a dairy herd where the virus was spreading slowly over more than 2 years and where there was no direct contact with PI animals, a SIR (susceptible/infectious/removed) model was used to determine the basic reproduction rate R_0 (the number of secondary cases produced by one infectious animal during the whole period in which it is infectious). Among groups of freely mixing cattle with no contact with PI animals, R_0 was estimated as 3.3 (CI: 2.6–4.1) (Moerman et al., 1993). There is no consensus on whether the transient infection will be an ongoing process or will be self-limiting and affect only a few animals (1992a). In some experimental studies, susceptible animals in direct contact with transiently infected animals did not get infected (Niskanen et al., 2000; Niskanen et al., 2002).

When PI animals are present in a herd there will be a continuous high infection pressure. Among 67 antibody-negative animals from herds with PI ani-

mals, retesting 6 months later showed that 65 had seroconverted (Houe and Meyling, 1991). This corresponds to an incidence risk of $65/67 = 0.97$ for a 6-month period and an annual incidence risk of: $1 - (1 - 0.97)^2 = 0.999$ i.e., practically a 100% risk of infection. The study by Moerman et al. (1993) also showed a 100% risk of seroconversion among groups of cattle having lived among PI animals. Among 22 cows that have had only a brief accidental contact with a PI animal, only 12 had become positive 4 months later (Moerman et al., 1993). Therefore the incidence risk naturally depends on the length of exposure to PI animals. Among 22 heifers brought in contact with a PI animal, all seroconverted within 5 months, with the fastest seroconversion among heifers with the closest contact to the PI animal (Wentink et al., 1991). It is important to stress that in production systems in which subgroups of the herd are segregated from PI animals, these subgroups of animals can stay uninfected for long periods (Taylor et al., 1997). In addition to variation in the production system, there are considerable differences in the pasturing conditions among countries. In Switzerland, animals are often pastured in the Alps where incidence risk during the summer for replacement animals has been calculated as 45% (Braun et al., 1998). On pastures with PI animals the incidence varied between 33–100% as compared to 6–22% on pastures without PI animals.

Antibody levels in bulk tank milk were taken at 1-year intervals among dairy herds in Sweden. It was found that 5 of 43 herds and 7 of 91 herds with an initial low level showed an increase in antibody level 1 year later, indicating that these herds had experienced a new BVDV infection during the year (Niskanen, 1993; Niskanen et al., 1995). The corresponding annual herd incidence risk can be calculated as 12% and 8%, respectively, for these herds. Among 90 dairy herds in Northern Ireland, with an initial low bulk tank milk antibody level, 12 had a substantial increase after 1 year, corresponding to an annual incidence risk of 13.3% (Graham et al., 2001). If herds with a smaller increase in antibody level were also included, the annual incidence risk could have been as high as 47.7%, but it was not known whether some of these moderate increases in antibody level were due to purchase of antibody-positive animals. In Denmark, repeated serological examination of individual dairy cattle in 9 and 24 herds revealed herd annual incidence risks of 52% and 26%, respectively (Houe and Palfi, 1993; Houe et al., 1997). Based on the annual tested antibody level in bulk tank milk, the average seroconversion

risk in Norwegian herds was estimated as 12% in 1993 (i.e., at the start of the eradication program) declining to 2% in 1997 (Valle et al., 2000a).

The incidence of birth of PI animals will naturally follow the incidence of transient infection in susceptible pregnant cows. Based on the incidence of transient infection and age distribution of cattle at the time of conception in a high-prevalence area, the theoretical incidence risk of fetal infection in the first 3 months of pregnancy was calculated as 3.3% (Houe and Meyling, 1991). Because of fetal death and abortions, the incidence of PI animals born would be somewhat lower. Although the incidence risk may be assumed to be relatively constant in a larger population (i.e., the infection is endemic), there is often high variation over time in the individual herd.

Some possible reasons for this variation are differences among BVDV strains (e.g., differences in viral shed or differences in PI/abortion ratios) and subsequent introduction of other BVDV that are antigenically divergent from the first BVDV. In addition PI animals often occur in clusters. In 22 dairy herds with a total of 129 PI animals, the incidence of PI animals born was closely related to the time of birth of the first PI animal (i.e., oldest identified PI animal) in the herd (Houe, 1992a). Thus 26.3% of the PI animals were born within the first 2 months after the oldest PI animal, whereas no PI animals were born from 2 months until 6 months after the oldest PI animal. Hereafter from 6–14 months after birth of the oldest PI animal, a larger group consisting of 52.7% of all identified PI animals was born (note that these percentages are compared to other PI animals and therefore not incidence risks, but a measure of relative incidence of PI animals born).

This pattern of occurrence of PI animals seems to reflect two periods of transient infection among cows: an initial period with transient infections of short duration (and there are probably no PI animals around) and a second period of transient infections of longer duration caused by continuous presence of PI animals. It is not possible to calculate the levels of incidence risk from these figures because the number of susceptibles at different periods was not known. Taken together, these studies indicate that at the herd level a common scenario concerning incidence could be the following:

- An initial transient infection period of relatively low infection pressure and variable duration (sometimes it stops and sometimes it will continue)

- After the birth of PI animals from 6 months later the incidence increase to high levels staying there as long as PI animals are present.

PREVALENCE AND INCIDENCE

Most epidemiological studies on the occurrence of BVDV infection have focused on determining the prevalence from cross-sectional or repeated cross-sectional studies. Studies on incidence are usually more expensive because it is necessary to take samples at more than one time point. However, under certain circumstances, the incidence can be calculated from data on antibody prevalence. The assumption is that the prevalence at a certain age is a measure of the incidence of infection throughout the cow's life until that age (Houe and Meyling, 1991; Toft et al., 2003). We, therefore, need a formula to sum up the I_{risk} over the animal's life. As calculation of incidence over sequential periods is dependent on the previous periods, it is easier to calculate the probability of avoiding infection over several periods and then finally subtract that probability from 1. The probability of avoiding infection in a given time period is $1 - I_{\text{risk}(i)}$ and the total probability of avoiding infection during n time periods is simply the product of each time period:

$$\text{Probability of avoiding infection} = \prod_{i=1}^n (1 - I_{\text{risk}(i)})$$

where \prod denotes the products of all time periods.

Hereafter the total incidence simply becomes the following:

$$I_{\text{risk total}} = 1 - \prod_{i=1}^n (1 - I_{\text{risk}(i)})$$

As the prevalence at a given age is a measure of total incidence risk and assuming a constant incidence over different time periods e.g., a constant annual incidence risk ($I_{\text{risk}(p)}$) the formula can be written as follows:

$$\text{Age specific prevalence} = 1 - (1 - I_{\text{risk}(p)})^n$$

where n is the number of time periods, each of the same length p —i.e., in this case the age in years. This methodology was used by Houe and Meyling (1991) where age specific prevalence of 0.48, 0.65, 0.75, 0.85, 0.95, and 0.96 for 1-year age groups from 1–7 years was entered on the left side of the formula together with age, whereafter the annual incidence risks were calculated as 0.35, 0.34, 0.32, 0.34, 0.42, and 0.39 for each age group, respectively. Thus it can be seen that the incidence was fairly con-

stant at around 0.34 for most age groups. It is an important assumption that the population is in an endemic situation with a constant infection pressure in the population as a whole. Similar increases in age-specific prevalence of antibody carriers by increasing age has also been seen in other studies (Harkness et al., 1978; Braun et al., 1998; Luzzago et al., 1999; McGowan and Murray, 1999).

RISK FACTORS FOR OCCURRENCE OF BVDV INFECTIONS

A *risk factor* can be defined as a factor that is associated with increased probability of a given event or outcome (infection, disease, or reduced productivity). Sometimes risk factors are used for all factors that have a statistical association with the outcome, but often they are used only for factors where a certain degree of causality can be anticipated. On the other hand, the term *etiology* is used for the immediate cause of the disease. The combination of etiology and risk factors defines the disease determinants, which are often described according to their association with the host, the infectious agent, or the environment. As the host and agent are described in detail in other chapters, this chapter will focus on the environmental determinants, which in this case are environmental risk factors.

Risk factors can be viewed as either the risk of infection or the risk of disease. For example, the use of common pasture will increase the risk of introducing the infection, whereas high humidity in the calf barn will increase the risk that calves develop pneumonia following infection. This section covers risk factors for the occurrence of infection, and the next section covers the effect of infection on disease and production (i.e., the risk of damages following infection).

When the outcome of risk factor studies is infection, disease, or other dichotomous variables, the prevalence of a given outcome (e.g., infection) in an exposed group can be compared to the prevalence in a nonexposed group by setting up the data in a 2×2 table:

Risk Factor Status	Infection Status		Total
	Infection	No Infection	
Exposed	a	b	a + b
Not exposed	c	d	c + d
Total	a + c	b + d	a + b + c + d = n

The risk in the exposed group (i.e., prevalence among exposed: $a/(a + b)$) as compared to the risk in the unexposed group (i.e., prevalence among non-exposed: $c/(c + d)$) is measured as the relative risk (*RR*):

$$RR = \frac{a/(a+b)}{c/(c+d)}$$

The *RR* is a measure of the relative importance or association of the risk factor with infection. If we want to measure the additional importance of the risk factor we simply subtract the prevalence in non-exposed group from the prevalence in the exposed group obtaining the so called attributable risk (*AR*):

$$AR = \frac{a}{a+b} - \frac{c}{c+d}$$

Another important measure is the odds ratio (*OR*). Rather than establishing the relation between prevalence in exposed and nonexposed group as for *RR*, the *OR* establishes the relationship between the odds in the exposed versus nonexposed group:

$$OR = \frac{a/b}{c/d} = \frac{a \times d}{b \times c}$$

Note that if a and c are small (low prevalence of infection), the *OR* is a good approximation of the *RR*. Usually the *RR* is preferable to *OR*, but as the *RR* is not a valid measure in case-control studies it becomes an important measure.

Risk factor studies on BVDV infections have often been performed as observational studies where the main types are cross-sectional, cohort or case-control studies. In cross-sectional studies we sample without regard to infection or exposure status, in cohort studies we sample according to risk factor status (only among noninfected), and in case-control studies we sample according to infection status. In case-control studies where we have sampled according to infection, we are not allowed to establish the prevalence measures of infection. Instead, prevalence measures or odds of risk factor relative to infection status can be calculated. Especially the odds for risk factor status are useful as the *OR* for risk factors ends up being the same formula as for *OR* for infection status:

$$OR_{\text{risk factor}} = \frac{a/c}{b/d} = \frac{a \times d}{b \times c}$$

Still, one should be more careful interpreting causality from *OR* obtained in case-control studies as it

measures the *ORs* of exposure. Thus, if infected animals in a case-control study have an *OR* of 2.0 of having been on the pasture, this is not the same as to say that pastured animals have an *OR* of 2.0 of being infected, although we intuitively feel that in practice it will almost be the same. There are several other measures on the importance of risk factors. For more detail, the reader is referred to epidemiological textbooks. Among the risk factor studies on BVDV the *OR* seems to be the most frequently used measure of effect.

The presence of PI animals in the vicinity of susceptible animals poses the highest proven risk of seroconversion in the population. In a cross-sectional study, the mean antibody prevalence among cattle from herds with PI animals was 87% as compared to 43% in herds without PI animals (Houe and Meyling, 1991). The *RR* of being infected according to the risk factor of coming from a herd with PI animals was not stated but it can be obtained simply as the relationship between the two prevalences ($87/43 = 2.0$) or to illustrate the use of the *RR* formula directly from the distribution of antibody carriers in the two herd categories:

Risk Factor Status:	Antibody Status of Individual Animals		
	Antibody-Positive	Antibody-Negative	Total
Presence of PI Animals in the Herd			
PI animals present	1083	166	1249
PI animals not present	572	749	1321
<i>Total</i>	1655	915	2570

Hereafter the *RR* is calculated as follows:

$$RR = \frac{a/(a+b)}{c/(c+d)} = \frac{1083/1249}{572/1321} = 2.0$$

This may be an underestimation of the effect of PI animals because many of the animals in herds without PI animals at the time of cross-sectional study may have been exposed to PI animals previously.

The following description of other risk factors will often be a reflection of the increased risk of direct or indirect contact with PI animals and to some extent with acutely infected animals or other sources. Some may claim that the presence of PI animals should be viewed as the direct etiology and not a risk factor, and often the distinction between

etiology and risk factor is unclear. However, this is more of an academic discussion.

The importance of different risk factors will vary substantially between regions. For example, the purchase of animals without testing will be a much higher risk factor in high-prevalence areas such as central Europe than in low-prevalence areas such as Finland. The following examples on risk factors should therefore be seen in the context of the prevalence, as shown in Tables 2.1–2.3.

In a cross-sectional study, the prevalence of antibody carriers was significantly higher among animals kept in extensive production systems (76.8%) than those in intensive production systems (70.9%) (Reinhardt et al., 1990). This was believed in part to be due to a higher rate of animal movement in extensive production systems. In the same study there was no difference in prevalence between beef, dairy, or mixed farming.

A case-control study of 314 herds in Norway compared risk factors between herds with antibody-positive young stock (case herds) and those with negative bulk tank milk samples (control herds) (Valle et al., 1999). A high number of risk factors occurred in a higher percentage of case herds as compared to control herds, including (percentage of case herds compared to control herds given in parenthesis): heifers on common pasture (21.0 vs. 8.7), sheep in pasture with cattle (31.5 vs. 22.1), breaking through pasture fences (33.8 vs. 24.6), over pasture fence contact (50.0 vs. 31.2), mixing of herds in pasture (43.8 vs. 30.6), wild animals in pasture (92.2 vs. 84.5), purchasing animals (67.3 vs. 48.7), not asking information about BVD when purchasing animals (73.4 vs. 40.9), other animal traffic including common summer housing and exchange of calves (9.6 vs. 0.6), veterinarian reusing needles between farms (7.1 vs. 1.3), and not using dairy advisors (25.0 vs. 12.0). However, due to the long list of variables and occasional low number of herds with the indicated risk factor, few risk factors were significant. In the logistic regression models, purchasing animals (OR = 1.8), use of common pasture (OR = 5.1), over pasture fence contact (OR = 2.5), purchasing without BVD documentation (OR = 5.4), not using dairy advisors (OR = 4.1), being a younger farmer, and “other animal traffic” (OR = 28.6) were found to be risk factors, which explained 51% of the seropositive herds.

In a repeat cross-sectional study in Denmark, 41 dairy herds were classified into lightly infected (≤ 3 antibody carriers among 10 young stock) and highly infected (≥ 8 antibody carriers among 10 young

stock) in 1992 and 1994 (Houe et al., 1997; Houe, 1999). Of the 24 herds being lightly infected in 1992, 11 changed to highly infected in 1994 and 13 remained lightly infected. The change in infection status was associated with the purchase of new animals within the past 3 years ($P = 0.052$) and moderately associated with pasturing cattle at a distance of less than 5 m to cattle from other herds or having other contacts with cattle from other herds ($P = 0.085$).

Risk factors for the presence of PI animals in cattle herds have been studied including demographic information on herd sizes and information from geographic information systems concerning distances between herds. Multivariable logistic regression with data from more than 8,000 herds showed significant effect of the following risk factors (OR, CI, and p values given in parenthesis) on the occurrence of PI animals: herd size measured as number of cows (OR = 1.09 per change in herd size of 10 cows; CI = 1.06–1.11; $P < 0.001$), mean distance to neighboring herds (OR = 0.87 per change in unit of 500m; CI = 0.81–0.93; $P < 0.001$) and sum of infected neighbors (OR = 1.54 for herds with > 3 infected neighbors as compared to no infected neighbors; CI = 1.17–2.02; $P = 0.024$ for the overall effect of sum of infected neighbors) (Ersbøll and Stryhn, 2000). In a study in U.S. herds with PI animals were significantly ($P < 0.01$) larger than herds without PI animals (Wittum et al., 2001). Thus, herds with PI animals had a medium number of 245 calves born in the calving season as compared to 89 in herds without PI animals. The OR for a herd of having high levels of antibodies in bulk tank milk was shown to increase by a factor 1.003 for each additional cow in the herd (Paton et al., 1998).

Several risk factors are confounded among each other. If, for example, larger herds purchase more animals than smaller herds then an apparent effect of herd size may in reality be due to purchase of animals and not to the herd size. It is, therefore, important that risk factor studies are performed by including several risk factors. Furthermore, there is a need to address interactions between risk factors. For example, the risk of infection at pasture will be higher in a high-prevalence area as compared to a low-prevalence area.

An alternative to large-scale studies identifying risk factors for the presence of infection is to perform intensive follow-up in recently infected herds. Follow-up investigations were performed in 67 previously BVDV-free Danish herds that got infected in 1998. Obvious explanations for re-infection were

identified in 74% of the cases. Of the 67 herds, 28% had purchased pregnant animals that later delivered PI animals. In 36% of the herds, PI animals had been present on neighboring pastures. Seven percent of the herds had had animals on common pasture and in 3% of the herds there had been PI animals in neighboring farmhouses. In the remaining 26% of the herds, no obvious explanations could be identified (Bitsch et al., 2000). Because this is an explorative type of study, probabilities cannot be given for the actual reasons apart from the given frequency distribution of the most obviously identified reasons.

As previously stated, the effect of risk factors will be highly influenced by the prevalence of infection in the area. Therefore the effect of different risk factors will obviously change during an eradication phase. It can be beneficial to combine different studies in order to obtain sufficient observational units for statistical analysis. Attempts have been made to compare risk factors in a low-prevalence area (Michigan) with that in a high-prevalence area (Denmark) (Houe et al., 1995a). The actual prevalence related to two previous studies (Houe and Meyling, 1991; Houe et al., 1995b; prevalence can be seen in Tables 2.1 and 2.2) showed that the prevalence of PI animals was 10 times higher in Denmark than in Michigan. When the effect of purchasing any animal versus no purchase of animals was analyzed separately in the two areas there was no significant effect of purchase on the occurrence of PI animals in individual herds. However, when including both areas in the analyses stratifying on area, a significant effect of purchasing animals was found. The analyses also included a number of demographic variables (either obtained at the country or state level or obtained for the studied herds), and all risk factors except herd size were in favor of the lower prevalence in Michigan. The following examples where the variables for Denmark versus Michigan are given in parentheses illustrate the differences: concentration of cattle in the country or state (DK: 67 per km², MI: 27 per km²), concentration of herds in the country or state (DK: 1 per km², MI: 0.29 per km²), herd size among the studied herds (DK: 135, MI: 274), use of pastures (DK: 79% of herds, MI: 45% of herds), purchase and addition of animals to the studied herds (DK: 18.5% of herds; MI: 12.6% of herds), and use of vaccination (DK: no vaccination, MI: 75% of studied herds). On a regional basis a study in England and Wales showed a significant relationship between antibody level in bulk tank milk and cattle population density (Paton et al., 1998).

With the high number of prevalence studies performed in recent years it would be an obvious next step to gather all the information on prevalence studies with information on cattle demographics (e.g., cattle density, herd density, herd size, trade patterns, use of vaccination) from a number of regions and perform formal meta-analyses of possible risk factors. Already some trends are obvious (Tables 2.1–2.3). For example, low-prevalence areas such as Norway and Finland have lower cattle population density and smaller herd size than high-prevalence areas (or areas that used to be high-prevalence areas before an eradication program), such as Belgium, Germany, and Denmark.

Despite the intuitively obvious effects of many of these risk factors, the documentation in literature is often scarce and the risk factors can explain only a relatively low percentage of infections. Some of the reasons for difficulties in establishing the risk factors may be due to uncertainties of the time of infection. For example, presence of infection in a herd may be due to introduction of infection 3–4 years ago as illustrated in the following study. Routinely recorded data (register data) from the Danish Cattle Database and the BVDV eradication scheme were used to estimate the origin of the first PI animal occurring in Danish cattle herds (Alban et al., 2001). The study showed that among herds participating in the milk recording scheme (i.e., dairy herds) the first PI animal was born to a dam that had been in the herd the whole period of lactation in 76.1% of the cases, PI animals were introduced directly in 4.5% of the cases and they were introduced by purchase of a dam in late gestation in 4.3% of the cases. In the remaining cases the exact source could not be identified. For comparison, among herds not in the milk recording scheme (mostly beef herds), the first PI animal was born from a dam that had been in the herd during the whole lactation in 26.3% of the cases, whereas PI animals had been directly introduced in 33.9% of the cases and through dams in late lactation in 2.4% of the cases. It should be noted that some of the dams that had been in the herds throughout their whole lactation could have been infected by previously purchased but unidentified PI animals. Still, most of the PI animals in the herds were generated within the herd, meaning that the exact trace back for risk factors often needs to go several years back in time. Therefore, cohort studies linking the time of infection with the time of occurrence of risk factors would be useful to further quantify the importance of risk factors.

QUANTIFICATION OF THE EFFECTS/CONSEQUENCES OF BVDV INFECTIONS ON DISEASE AND PRODUCTION

The measures of consequences include the presence of the disease as well as reduced production. The clinical manifestations often used in epidemiological studies to measure the effect of transient infection include diarrhea, reduced milk production, reproductive disorders, increased occurrence of other diseases, and death. Losses from fetal infection include abortions, congenital defects, birth of weak and undersized calves, unthrifty PI animals, and death among PI animals (Baker, 1995). These consequences of BVDV infections have been documented in several experimental and case studies, whereas quantification of the consequences in larger observational studies is often less well documented. The validity of these data in relation to their verification of being BVD-related varies substantially. The losses following acute (transient) infection are especially difficult to quantify. Losses such as respiratory disease, repeat breeding, or abortions have seldom been measured together with a rise in antibody titer, and therefore such losses are often analyzed in retrospective studies, where the time period of transient infections is only loosely estimated. Losses among the PI animals have been quantified with a higher degree of certainty because the presence of persistent infection together with clinical symptoms has often been documented. Measures of prevalence and incidence together with quantification of disease and production parameters are the foundation for calculating the effect of BVDV in economical terms. The following sections emphasize measures of consequences that have been quantified under field conditions followed by estimation of economic impact of BVDV infections. On some occasions quantitative assessments from case studies and experimental studies are also given when they are judged to have sufficient quantitative interpretation.

The effects of infection have often been investigated in case studies and experimental studies, but to a lesser extent in observational studies. All of these approaches have drawbacks associated with them. For example, more severe outbreaks tend to attract attention and are liable to be investigated and published leading to overestimation of the effect of BVDV infections. In experimental studies, it is difficult to mimic natural circumstances and it has often been difficult to demonstrate clinical signs of acute BVD, although reproductive disorders (e.g.,

congenital defects) have frequently been documented. Observational studies often suffer from the lack of knowledge of how many animals actually seroconverted and at what time points. Therefore, the effects seen in observational studies are probably underestimated when considering the effect in the individual seroconverting animal. However, observational studies are useful for estimation of the effect of infection in a partly immune population.

SUBCLINICAL INFECTIONS

The clinical spectrum of postnatal infection of immunocompetent animals ranges from subclinical infection to severe disease followed by death. Several years ago it was estimated that 70–90% of transient infections were mild or subclinical (Ames et al., 1986). Although the risk of disease following transient infection has not been exactly quantified, epidemiological investigations indicate that these estimates are good. The clinical consequences of acute BVDV infection were studied in a large dairy herd where BVDV was circulating for approximately 2.5 years (Moerman et al., 1994). Among 136 cattle with transient infection, 129 (95%) showed no clinical signs of infection, 5 animals were slightly affected, and only 2 animals were severely ill. Furthermore, many studies in the 1990s have shown that BVDV infection was more widespread than previously thought, indicating the presence of a large number of subclinical infections.

REDUCED MILK YIELD

Milk yield was studied in a longitudinal study of 54 cows of which 22 seroconverted (Moermann et al., 1994). A gradual drop in the milk yield (measured as moving average over 3 days) of 10% or more within 10 days was seen in 18 out of 22 cows that seroconverted and 9 out of 32 cows that did not seroconvert, giving an OR of reduced milk production of 11.5. On the other hand comparison of antibody level in bulk tank milk and milk yield did not reveal a significant association (Graham et al., 2001).

OCCURRENCE OF OTHER DISEASES FOLLOWING TRANSIENT INFECTION IN COWS

Examination of bulk tank milk samples from 213 Swedish dairy herds in 1988 and 1989 indicated that recent infection had occurred in 7 herds (Niskanen et al., 1995). Compared to 84 herds with continuous low level of antibodies in bulk tank milk (i.e., uninfected), cows in the 7 infected herds had increased ORs for several clinical conditions, with mastitis,

miscellaneous diseases, and retained placenta being significant (OR and 95% CI for OR are given in parenthesis): mastitis (OR = 1.8; CI = 1.1–2.8), tramped teat (OR = 1.6; CI = 0.9–2.8), ketosis (OR = 1.3; CI = 0.6–2.2), miscellaneous diseases (OR = 2.8; CI = 1.7–4.4) and retained placenta (OR = 2.8; CI = 1.6–4.7). Increased risk of retained placenta may also be seen among dams delivering PI calves (i.e., several months after the transient infection). Thus, 5 (41.7%) out of 12 PI dams had retained placenta compared to 7 (3.5%) cases of retained placenta in 198 non-PI dams ($P < 0.001$) (Larsson et al., 1994).

In Norway, the national screening of all dairy herds for BVDV antibodies in bulk milk in 1992 and 1993 was used to estimate the effect of transient infection on udder health (Waage, 2000). A total of 404 herds with a significant rise in antibody level in bulk milk were compared to herds with continuous low levels of antibodies. In infected herds, there was a 7% (CI = 0.2%–11.4%) increase in the incidence of clinical mastitis in the year of exposure as compared to negative control herds but there was no significant effect on the bulk milk somatic cell count or the culling rate due to mastitis. However, a study by Graham et al. (2001) found a significant increase in somatic cell count with increasing antibody levels in bulk tank milk ($P < 0.01$).

OCCURRENCE OF OTHER DISEASES FOLLOWING TRANSIENT INFECTION IN CALVES

Transient BVDV infections have been associated with respiratory diseases and diarrhea in calves. However, due to the multifactorial nature of these diseases it is difficult to quantify the exact role of BVDV in their occurrence. Different field investigations indicate that BVDV will often double the risk of these conditions. Among calves entering a feedlot, 13 of 29 (45%) calves treated for respiratory disease and 8 of 36 (22%) untreated calves had seroconverted (Martin and Bohac, 1986). This corresponds to an OR of 2.8 for treatment following seroconversion. The effect of BVDV infection on the occurrence of respiratory diseases has also been demonstrated indirectly by comparing calves receiving colostrum from seronegative dams with calves receiving colostrum from seropositive dams (Moerman et al., 1994). Among calves exposed to BVDV soon after birth, 30 of 44 calves (68.2%) receiving colostrum from seronegative dams developed moderate or severe bronchopneumonia whereas 35 of 86 calves (40.7%) receiving colostrum from seroposi-

tive dams developed moderate or severe bronchopneumonia. This corresponds to an OR of 3.1 for developing bronchopneumonia if receiving colostrum from antibody-negative dams. In another herd 10 of 61 calves (16.4%) born in the period when BVDV was introduced into the herd, underwent veterinary treatment for respiratory disease and/or enteritis between 2 days and 4 months of age, whereas only 5 out of 134 calves (3.7%) born before or after the introduction period received treatment ($P < 0.01$) (Larsson et al., 1994). This corresponds to an OR of 5.1 of being treated if born in the period of BVDV introduction. In the same study the mortality was 13.1% among calves born in the period of introduction of BVDV as compared to 2.2% in the other periods ($P < 0.01$).

After strict closure of a dairy herd together with eradication of BVDV the incidence of diarrhea among calves in the first 31 days of life decreased significantly from 70.6% to 19.4% (Klingenberg et al., 1999). However, it was difficult to distinguish the strict effect of BVDV compared to general improvement in management practice including closure of the herd.

In addition to having an effect on clinical diseases, BVDV also has an effect on the occurrence of other infections. For example infectious bovine rhinotracheitis (IBR) virus was much more widespread in the respiratory tract of calves previously exposed to BVDV (Potgieter et al., 1984a). Furthermore, there are many examples that BVDV will aggravate the severeness of other infections—for example, *P. haemolytica* (Potgieter et al., 1984b; also see Chapter 9 on immunity and immunosuppression). However, the quantitative effect of BVDV on the prevalence, incidence, and severity of other infections is not completely documented.

TRANSIENT INFECTION BY VIRULENT STRAINS OR BVDV IN COMBINATION WITH OTHER PATHOGENS

For many years postnatal infection of immunocompetent animals was characterized as mild or subclinical in most cases. In the 1990s there came increased evidence of the importance of highly virulent strains with much more severe consequences following transient infection (hemorrhagic syndrome and peracute BVD) especially with BVDV genotype 2. Although there is no unambiguous connection between genotype and virulence, genotype 2 has predominated as the cause of hemorrhagic syndrome and peracute BVDV (Bolin and Ridpath, 1996; Ridpath et al., 2000).

An outbreak of acute BVD in a herd also infected with *Leptospira hardjo* and *Coxiella burnetii* caused very severe losses (Pritchard et al., 1989). In a dairy herd with 183 milking cows, the losses included 15 dead cows, 20 culled cows (primarily because of emaciation and infertility), 40 abortions, 18 stillborn calves, and 3 calves dying soon after birth. Other case descriptions of acute outbreaks have revealed morbidity of up to 40% and mortality up to 10% (Hibberd et al., 1993; David et al., 1994).

In 1993, BVDV genotype 2 was associated with severe outbreaks of BVDV in Ontario, Canada. A study of seven herds (mean number of cattle: 93; range: 40–191) with outbreaks of acute BVD revealed a mortality among adults of 9% (range 2–26%), a mortality among immature animals (<2 years of age) of 53% (range 13–100%), and 44% (range 3–83%) mean abortion frequency based on the number of breeding age females. Furthermore, the occurrence of respiratory diseases was high along with a number of sequelae (Carman et al., 1998). In 1993, the mortality was estimated as 31.5% for grain-fed calves and 17.1% for milk-fed calves, and the overall mortality was 32,000 out of 143,000 calves (Pellerin et al., 1994).

REPRODUCTIVE DISORDERS

Reproductive disorders are a very important clinical manifestation. These are outlined in more detail in Chapter 8. This chapter emphasizes the quantitative aspects of their occurrence. An often-used measure for reproduction efficiency is the conception rate. Note in the following examples that although the measure is usually defined as a proportion it is still termed *conception rate* (although it is mathematically not a rate). In an experimental study, 15 heifers (Group I) were infected by contact with PI animals 4 days after insemination, another 18 heifers (Group II) were infected intranasally 9 days before insemination, and 14 uninfected heifers acted as control group (McGowan et al., 1993). The conception rates 20 days after insemination (defined as proportion of eligible heifers that had serum progesterone concentration of more than 2.0 ng/ml on day 20 after insemination) were 60% (9 of 15) and 44% (8 of 18) for Groups I and II as compared to 79% (11 of 14) in the control group. At 77 days after insemination the conception rates (defined as proportion of eligible heifers which were palpably pregnant 77 days after insemination) were 33% (5 of 15) and 39% (7 of 18) for the infected groups; those for the control group remained unchanged. At 77 days, conception rates in Groups I and II were both significantly

lower than the control group. A group of seronegative cows that were inadvertently exposed to a PI animal were bred before (9 cows), during (9 cows), or after seroconversion (14 cows) (Virakul et al., 1988). The first service conception rates at 21 days in these three groups were 22.2%, 44.4%, and 78.6% (the difference between 22.2% and 78.6% being significant at $P < 0.05$).

The severe effect on conception rates is also seen in observational studies, although the effect is somewhat less pronounced. In five Danish dairy herds, the overall conception rate in a risk period of infection (presence of a young PI animal spreading the infection) was 38% as compared to a conception rate of 47% ($P < 0.001$) in a post-risk period (presence of older PI animals where the herd would be immune) (Houe et al., 1993a). The increase in conception rate ranged from 16–64% in individual herds.

In seven Swedish herds with recent infection there was an increased OR of estrus stimulating treatments (OR = 2.2; CI = 1.0–4.9); however, the number of inseminations per service period was not significantly increased (Niskanen et al., 1995). In Norway the ORs for abortions in herds with recent infection was calculated as 2.6 and 11.6, respectively for two different registration periods ($P < 0.01$) as compared to BVDV free control herds (Frederiksen et al., 1998). A Swiss study showed that animals seroconverting in midgestation (days 46–210) had an OR of abortion of 3.1 ($P = 0.02$), whereas an effect was not seen after infection in later stages of gestation (Rüfenacht et al., 2001). In the study by Moerman et al. (1994), abortions were not more frequent in seroconverting cattle. A field investigation of the causes of abortions in the United Kingdom showed that 39 out of 149 (26%) abortions in 54 farms were associated with BVDV (Murray, 1990).

Although congenital defects, stillbirths and weak-born calves have been demonstrated in many experimental studies (e.g., Done et al., 1980; Liess et al., 1984; McClurkin et al., 1984; Roeder et al., 1986), they have seldomly been quantified in larger observational studies. Some observational studies did not find significantly more stillbirths, weak-born calves, or congenital defects (Moerman et al., 1994; Frederiksen et al., 1998; Rüfenacht et al., 2001).

LOSSES AMONG PI ANIMALS

PI animals are often born weak and undersized and when they grow up they are at risk of acquiring other diseases and being culled due to unthriftiness or they may die from mucosal disease. In a study the

mean heart girth at 80 days of age among 8 male PI calves was 96 cm as compared to 100.5 cm among 13 male non-PI calves ($P < 0.05$) (Larsson et al., 1994). The same figures at 180 days were 123.3 vs. 130.2, respectively ($P < 0.05$).

The incidence risk of dying or being slaughtered due to unthriftiness within 1 year among 34 PI animals in 10 Danish dairy herds was 0.28 and 0.31, respectively (Houe, 1993). The attributable risk for PI animals of leaving the herd for any reason within 1 year was calculated as 0.35. In eight Danish dairy herds with outbreaks of mucosal disease, 31 of 52 PI animals died that were identified as PI either at outbreak or following whole herd testing (Houe et al., 1994). However, as these were selected outbreaks, this mortality might have been higher than what would occur among PI animals in the whole cattle population. On the other hand, had whole herd testing not been performed more PI animals would probably have died before they were identified as PI animals and slaughtered. Another way of judging the mortality among PI animals is to look at age specific prevalence. Thus, the prevalence of PI animals among all cattle in 19 Danish dairy herds was 1.4% (Houe and Meyling, 1991). However, among cattle younger than 1 year of age the prevalence was 2.9% indicating a high mortality (or at least culling) also among younger PI animals. In a prevalence study by Frey et al. (1996), 66% of the PI animals were younger than 1 year, whereas only 10% were older than 2 years.

ECONOMIC IMPACT OF BVDV

Economic calculations and models depend on the underlying data. The types of data necessary for calculation of economic impact have been outlined in previous sections of this chapter. The significant variation in occurrence, frequency, and damaging effects following infection will affect the uncertainty in economic estimations. Also, there are indications of a changing disease pattern of BVDV over time (Evermann and Ridpath, 2002) and hence incorporation of appropriate variables in the economic models should be a dynamic process.

There are two major functions of performing economic analyses. First, calculation of the pure costs of disease will give an indication of the relevance of disease as compared to other diseases in animal husbandry. In addition to economics, there are other criteria for ranking the relevance and importance of diseases, such as animal welfare and human safety. These criteria will not be discussed further although animal welfare is an important aspect of BVDV in-

fections due to the severe suffering from mucosal disease. The second purpose of performing an economic analysis is to guide decision makers in determining whether it is cost-effective to control the infection and also to compare the cost effectiveness of different control options. The different control options are discussed in Chapter 14.

The direct economic cost (C) of a disease is the sum of the production losses (L) and the expenditures (E) for treatment and prevention (McInerney et al., 1992; Bennett et al., 1999a):

$$C = L + E$$

The expenditures for treatment and prevention are often relatively easy to obtain as they follow specific actions in the herd. The production losses on the other hand are often associated with much higher uncertainty due to biological variation. The production losses can be obtained if there is general information about the population size, the incidence of infection, damaging effect (including both the probability and magnitude) on disease and production, and the value of each damaging effect:

$$L = \sum \text{population size} \times \text{incidence} \times \text{effect} \times \text{value}$$

The calculations need to be done for each damaging effect and the associated value (drop in milk production, abortion, death, etc.).

The economic losses at the herd level are often determined from what actually happened in a particular herd or group of herds. In larger populations and at the national level, it is more common to first establish the relevant variables (population size, incidence, effect, and values) and then include them in economic models. The following section gives some examples of economic estimations that have been published. The size of the economic estimations is cited as they appeared in the original publications, sometimes together with recalculations in other publications. In addition, if standardization has been made (e.g., measuring economic values per cow), this is also included.

Present values (PV) can be calculated based on earlier values (EV) and the real rate of interest (r), where the real rate of interest is the difference between the market rate of interest and the inflation rate (Huirne and Dijkhuizen, 1997). The formula for calculating the present value based on a given earlier value n years ago is

$$PV = EV \times (1 + r)^n$$

In addition, the exchange rate between currencies should also be considered.

ECONOMIC LOSSES AT THE HERD LEVEL

There are many ways to estimate the economic impact at the herd level in any given outbreak situation. The outbreak situation can consist of accumulated clinical data of both reproductive disorders and losses among PI animals over a longer time period, or they may only estimate the sudden losses of an outbreak of mucosal disease over a few months. In any case they usually reflect observable problems. Often only outright losses are included.

In an outbreak of abortion, neonatal death and subsequent mucosal disease in a 67-cow dairy herd the estimated economic losses varied between £1720 and £4115, depending on whether dead or culled animals were replaced (Duffel et al., 1986). In U.S. dollars this corresponds to approximately \$40–95 per dairy cow. A study of 14 outbreaks in the Netherlands including losses due to abortions, stillbirths, various clinical lesions, and mucosal disease had losses in the range of 42Dfl. to 285Dfl. (\$24–161) per dairy cow (Wentink and Dijkhuizen, 1990). In outbreaks of mucosal disease in eight Danish dairy herds, the losses due to mucosal disease only were estimated to be \$13–39 per animal corresponding to \$33–98 per dairy cow (Houe et al., 1994).

Case descriptions of severe outbreaks of acute BVD have resulted in losses several times higher. An outbreak with a combined infection with BVDV, *Leptospira Hardjo* and *Coxiella burnetii* was, associated with death in adult cows, abortion, and neonatal mortality. This resulted in a total cost of more than £50,000 (Pritchard et al., 1989) corresponding to \$410 per cow. Seven outbreaks of severe acute BVD in Canada with high mortality and abortion rate caused losses in the range of \$40,000–100,000 per herd (Carman et al., 1998). These herds had between 40 and 191 animals, so the losses per cow would be higher than \$400 per cow.

This means that whether we are dealing with a more “classic” outbreak with losses in the range of \$50–100 per cow (note that these figures are per all cows being in the herd and not only diseased cows) or we are dealing with severe outbreaks of acute BVD with losses of more than \$400 per cow, the losses make up a significant percentage of the total value of the livestock. Due to the variation of these estimations and the uncertainty of how representative these cases are, we can only have rough indications of the true losses or costs. Therefore BVDV is among the diseases where an outbreak can be devastating for the economy of the individual farmer.

ECONOMIC LOSSES IN LARGER POPULATIONS AND AT THE NATIONAL LEVEL

The losses or costs for the cattle industry may be better calculated using average values for variables obtained from epidemiological studies rather than summing up the losses described in case studies. There are significant differences in the virulence of BVDV strains and both genotype 1 and genotype 2 have high virulent strains. We only have sparse information on the occurrence of strains with different virulence, so this information cannot be directly incorporated in the models for calculating the economic impact. Therefore, many calculations have been performed anticipating an average effect of infections within the area, or the estimations have been made anticipating either a high-virulent or a low-virulent strain being present.

Using the formula for production loss, the economic losses in Denmark before initiation of the eradication campaign have been estimated as \$20 million per million calvings (Houe et al., 1993b; Houe, 1999). Including the variables on damaging effects from virulent strains the losses were estimated as \$57 million per million calvings (Houe, 1999). These calculations were based on an annual incidence risk of 34% corresponding to very high prevalence of infection, and therefore the magnitude of the losses should be adjusted when considering areas with a lower prevalence (for details see Houe, 1999). The losses in Norway (at the start of the eradication program) were estimated as 26 million Nkr per year (Valle et al., 2000b) corresponding roughly to \$10 million per million calvings. This corresponds well with the lower prevalence seen in Norway as compared to the prevalence in Denmark. In Great Britain, the direct costs associated with BVDV have been estimated as between 5 and 31 million£ (1996 values) (Bennett et al., 1999b), which with an estimated 4.5 million calvings roughly corresponds to \$6 million per million calvings. In this case, the population at risk was considerably lower compared to the one used in the Danish model. In Canada, the total annual cost for an average 50-cow dairy herd have recently been estimated as \$48 per cow (Chi et al., 2002).

It is noteworthy that in a high-prevalence area the average loss due to infection is almost half the amount of losses calculated in the outbreak situation. But in many herds the losses were not very obvious because most herds were partly immune and abortions and deaths were more often seen as occasional cases than actual outbreaks. Therefore calcu-

lation of the economic losses has been an important motivator for considering control programs both when farmers have experienced outbreaks and when there was a more continuous occurrence of various losses.

OPTIMIZING DECISIONS BASED ON ECONOMIC CALCULATIONS

From an economical point of view the optimal situation is when the total costs are minimized—i.e., the sum of losses and expenditures are minimized, which is the basic economical principle (McInerney et al., 1992). Naturally, the more money we spend on treatment and prophylaxis the more the production losses will be reduced. However, we should only continue to increase expenses as long as \$1 spent on treatment and prophylaxis will cause a reduction in production loss of more than \$1. In practice, it is not always possible to change the size of the expenditures on a continuous scale because a control strategy (e.g., vaccination) will only make sense if it is performed to a certain extent. But still calculating losses and expenses for different control strategies makes them directly comparable from an economic point of view.

ECONOMIC EVALUATION OF CONTROL STRATEGIES AT THE HERD LEVEL

Decision tree analyses have been used to support decision of selecting a blood testing strategy following outbreaks of mucosal disease (Houe et al., 1994). The calculations showed that testing a risk group of animals being no more than 3 months younger or older than the age of the index case of mucosal disease was most beneficial to the greatest number of farms. However, the most beneficial strategy was always dependent on the individual farm. Furthermore, decision tree models are not suitable for handling the long-term effect of control decisions. The effect of different control strategies has been evaluated in simulation models. A model simulating Dutch conditions showed that culling of PI animals was unattractive (Pasma et al., 1994). However, the conclusion was highly dependent on the risk of reinfection. For comparison, a Markov Chain model assuming that reinfection could be avoided was in favor of eradication. Bennett (1992) described a decision support system showing that depending on the farm-specific situation, the recommendation could be any of the following: a do nothing strategy, a culling strategy, or a vaccination strategy. In conclusion, a general optimal control strategy cannot be given because it will depend on an evaluation of the

farmer's capability to avoid reinfection and certainly also of the general infection status of the area the farm is located in.

ECONOMIC EVALUATION OF NATIONAL ERADICATION PROGRAMS

In Norway, a cost-benefit evaluation of the control and eradication program from 1993 to 1997 was made by subtracting the program costs from the benefits (Valle et al., 2000b). The 1993 net present value for the 5 years of the program was calculated as \$6 million, and the program was cost-effective in the second year. For comparison, it was estimated that an eradication program in France would take 15 years to be cost-effective (Dufour et al., 1999). Before the eradication program was initiated, the annual losses in Denmark were estimated as approximately \$20 million. Thus, this amount is a possible benefit that can be obtained after total eradication. In the initial phases the cost of the program was approximately \$9 million per year (Bitsch and Rønsholt, 1995), thereafter being reduced to \$3.5 million per year. The costs for continuous monitoring have not been established but will be significantly lower. Because the country is close to total eradication, an annual benefit of almost \$20 million is obtained and the program can therefore be considered highly cost-effective. As for the individual herd level, the calculations will be highly dependent on the capability to stay free of infection. Another important factor for making the Scandinavian program very cost-effective is the low cost of using bulk tank milk testing for antibody for the continued monitoring of dairy herds.

CONCLUDING REMARKS AND PERSPECTIVES

This chapter summarizes results of epidemiological studies on the occurrence of bovine virus diarrhea virus (BVDV) infections, identification of risk factors, and effects of the infection on disease and production. The occurrence is described as prevalence and incidence of antibodies and virus, both at the animal level and at the herd level. It is concluded that laboratory methods are sufficiently valid at the animal level for their use in epidemiological studies. At the herd level the sensitivity and specificity show larger variation. Studies show that in many areas the prevalence of PI animals is 0.75–2% and that 60–70% of animals are antibody-positive. However, there may be regional differences in prevalence. For example, many studies in the U.S. have revealed a lower prevalence of PI animals than those reported

in European studies. Other differences in prevalence may be related to cattle density and management style. For example, some areas with low cattle population density and small herds seem to have relatively low prevalence.

Susceptible animals housed together with PI animals will have a very high incidence rate of infection, and almost 100% of the animals will be infected within a few months. In production systems in which subgroups of the herd are segregated from PI animals, these subgroups of animals can stay uninfected for long periods.

Risk factors for BVDV infections are often a reflection of the risk of direct or indirect contact with PI animals. Risk factors include livestock trade, pasturing of animals, use of common pasture, cattle density in the area, herd size, number of infected neighbors, fence breakout, other animal contact between herds (e.g., exhibitions), insufficient hygienic procedure, and occurrence of other species (sheep, wild life). The documentation in literature on the importance of risk factors is often surprisingly low as compared to the obvious effect one would anticipate. Often the proven risk factors explain only a relatively low percentage of infections. Some of the reasons for the difficulties of establishing the importance of risk factors may be due to uncertainties of the time of infection of herds. The improved surveillance systems for herds make larger risk factor studies possible in the future with more precise identification of the herd infection time. Furthermore many risk factors are confounded among each other increasing the need for studies on a larger scale. With the high number of prevalence studies being performed, it would be recommended to gather information on risk factors in a uniform way between studies in order to combine them in formal meta-analyses. These studies should also include information on cattle demographics (e.g., cattle density, herd density, herd size, trade patterns, use of vaccination).

Postnatal infection of immunocompetent animals is often subclinical in most animals. Some may show the typical clinical signs of acute BVD. Transient infection in cows may be followed by reduced milk production, higher incidence of other diseases—such as mastitis—ketosis, and retained placenta. Transient infection in calves may be followed by increased frequency of respiratory diseases and diarrhea. The infection can have significant effect on reproductive disorders, such as conception rate, abortions, congenital effects, and weak-born calves. PI calves are often weak and un-

dersized and have a considerable increased risk of either being culled early due to unthriftiness or dying throughout their lifetime. Different observational studies on the effect of infection show remarkable differences in the clinical consequences. Although some of the differences obviously seem to be due to differences in virulence, a closer understanding of the host-agent-environment triad seems relevant for the understanding of the variation. There is a high variation in the general disease level in herds without BVDV infection, and the importance of infection to herds with a general high health status as compared to herds with low health status needs to be clarified. A lot of the variation seen in the observational studies can also simply be due to chance. If for example the virus is spread at a time when there are many cattle in first trimester means different consequences compared to the situation where most cattle would be in the last trimester. Such differences would be most obvious in herds with seasonal calvings. Furthermore many observational studies are based on the fact that some infection took place, but without knowing exactly how many seroconversions occurred.

Estimations of economic impact are often attempted by combining all the information presented in this chapter, including the occurrence of the infection (prevalence and incidence) and quantifications on the different clinical and production consequences following infection. In order to improve the economic models, a closer description of distribution of the different genotypes and especially the differences in virulence both within and between genotypes would be needed.

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3

Classification and Molecular Biology

Julia F. Ridpath

TAXONOMY—DEFINING CHARACTERISTICS OF BOVINE VIRAL DIARRHEA VIRUSES

THE FLAVIVIRUS FAMILY

Bovine viral diarrhea viruses belong to the pestivirus genus within the Flavivirus family (Heinz et al., 2000) and, as such, possess many traits characteristic of this family. Like all flaviviruses, they have a lipid envelope that is derived from the membranes of the infected host cell. The lipid envelope makes these viruses susceptible to inactivation by solvents and detergents.

Similar to other flaviviruses, BVDV have a single-stranded positive sense RNA genome. The organization of the genome, which is conserved within the Flavivirus family, consists of a single open reading frame (ORF) flanked by 5' and 3' untranslated regions (UTR). The ORF is translated as a single polypeptide that is cleaved by viral and cellular proteinases into the individual viral proteins. The structural proteins are coded in the 5' end of the genome, and the nonstructural proteins are coded in the remaining 3' end of the genome. The replication of the RNA genome occurs in the host cell cytoplasm. The viral particles are assembled and enveloped at intracellular membranes, transported in cytoplasmic vesicles through the secretory pathway and released by exocytosis.

CHARACTERISTICS UNIQUE TO THE PESTIVIRUS GENUS

Viruses from the pestivirus genus have several characteristics that differentiate them from other member viruses of the Flavivirus family. Pestiviruses encode two proteins that are unique to their genus. The first is the nonstructural N^{pro}, which is the first protein coded by the pestivirus ORF. The N^{pro} is a proteinase whose only known function is cleaving itself

from the viral polypeptide. The second unique gene product is the E^{ns}. It is an envelope glycoprotein that possesses an intrinsic RNase activity.

Pestiviruses are also unique among flaviviruses in their resistance to inactivation by low pH. Most flaviviruses are inactivated by low pH, but pestiviruses are stable over a broad pH range (Hafez and Liess, 1972).

MEMBERS OF THE PESTIVIRUS GENUS

There are currently four recognized species within the pestivirus genus: **BVDV 1** (type virus BVDV 1a-NADL, accession #M31182); **BVDV 2** (type virus BVDV 2a-890, accession #U18059); **border disease virus (BDV)** (type virus BDV-BD31, accession #U70263); and **classical swine fever virus (CSFV)**, previously known as hog cholera virus (type virus Alfort/187, accession #X87939). The pestiviruses were originally classified into the species BVDV, CSFV, and BDV based on the animal host they originated from. This classification proved problematic because some pestiviruses are not restricted to a single host. Viruses characterized as BVDV, for example, were shown to infect cattle, sheep, and swine (Paton, 1995a, b). Subsequent efforts to divide pestiviruses into species based on monoclonal antibody (Mab) binding (Moennig et al., 1987; Bolin et al., 1988; Hess et al., 1988; Zhou et al., 1989; Edwards et al., 1991; Edwards and Paton, 1995; Paton et al., 1995b) had limited success due to antigenic cross reactivity between proposed species, antigenic variation within proposed species, and the ease with which Mab escape mutants could be generated. Phylogenetic analysis of genomic sequences has proved very helpful in differentiating pestivirus species (Hofmann et al., 1994; Harasawa, 1996; Becher et al., 1997; Ridpath and Bolin, 1997; Sullivan et al., 1997; Vilcek et al., 1997; Harasawa et al., 2000; Vilcek et al., 2001b). The comparison of genomic

sequence of viruses classified as BVDV led to the conclusion that there were actually two genotypes of BVDV, BVDV 1, and BVDV 2 (Pellerin et al., 1994; Ridpath et al., 1994a). The degree of sequence identity between pestiviruses in the 5' UTR region is the most frequently used parameter for differentiation pestivirus species (Ridpath et al., 1994a; Harasawa and Mizusawa, 1995; Harpin et al., 1995; Harasawa, 1996; Baule et al., 1997; Becher et al., 1997; Giangaspero et al., 1997; Ridpath and Bolin, 1997; Sandvik et al., 1997; Vilcek et al., 1997; Wolfmeyer et al., 1997; Harasawa and Giangaspero, 1998; Ridpath and Bolin, 1998; Shimazaki et al., 1998; Letellier et al., 1999; Sakoda et al., 1999; Flores et al., 2000; Ridpath et al., 2000; Falcone et al., 2001; Tajima et al., 2001; Tajima et al., 2001; Vilcek et al., 2001b; Beer et al., 2002; Couvreur et al., 2002; Evermann and Ridpath, 2002; Flores et al., 2002). However, differences between BVDV 1 and BVDV 2 strains are found throughout the genome (Ridpath and Bolin, 1995b, 1997).

All pestiviruses are antigenically cross-reactive. Although antigenic differences do exist between pestivirus species, they are not extensive enough for pestivirus species to be recognized as serotypes. Convalescent sera generated against a virus from a particular pestivirus species will generally have a higher titer against other viruses from that species as opposed to viruses from the other pestivirus species (Ridpath, 2003). However, animal-to-animal variation and divergence among viruses from the same pestivirus species can make it difficult to reproducibly and reliably differentiate pestivirus species based on serology alone (Muller et al., 1997; Bolin and Ridpath, 1998). Mabs have been developed that differentiate between the pestivirus species (Wensvoort et al., 1989a; Zhou et al., 1989; Dahle et al., 1991; Edwards et al., 1991; Kosmidou et al., 1995). However, cross-reactivity between pestivirus species and variation within any one species make it difficult to generate a Mab that simultaneously differentiates between species and still recognizes all the viruses within one species. Antigenic variation is particularly pronounced among BVDV 1 and BVDV 2 strains (Ridpath et al., 1994a) and impacts on both detection and control.

In addition to the four recognized pestivirus species, three putative species have been suggested. These putative species are based on viruses isolated from a giraffe, a reindeer, and a pronghorn antelope (Becher et al., 1997; van Rijn et al., 1997; Harasawa et al., 2000; Vilcek et al., 2001b). Only one virus has been isolated for each of these putative species,

and no correlation with clinical disease has been reported.

THE PESTIVIRUS VIRION

Pestivirus virions are enveloped, spherical particles that are 40 to 60 nm in diameter. The virions are made up of a central capsid, composed of the virally encoded C protein and the genome RNA, surrounded by a lipid bilayer. The capsid appears as an electron-dense inner core with a diameter of approximately 30 nm (Horzinek et al., 1971). The structure and symmetry of the core has not been determined. Three virus encoded proteins—E^{ms}, E1, and E2—are associated with the lipid bilayer envelope. The envelope surrounding the virion is pleomorphic, which impedes purification of infectious particles by banding in sucrose gradients and identification by electron microscopy. There appear to be 10–12 nm ring-like subunits on the surface of the virus envelope (Heinz et al., 2000). The Mr of the virion is estimated as 6.0×10^7 , and the buoyant density in sucrose is 1.10–1.15 gm/cm³ (Heinz et al., 2000).

Virions are stable within a pH range of 5.7 to 9.3 (Hafez and Liess, 1972). Infectivity is not affected by freezing but decreases at temperatures above 40°C (Heinz et al., 2000). Like other enveloped viruses, BVDV are inactivated by organic solvents and detergents. Other methods of inactivation include Trypsin treatment (0.5 mg/ml, 37°C, 60 min) (Liess, 1990), ethylenimine (reduction of $5 \log^{10}$ units using 10 mM at 37°C for 2 h) (Preuss et al., 1997), electron beam irradiation (4.9 and 2.5 kGy needed to reduce virus infectivity $1 \log^{10}$ unit for frozen and liquid samples respectively) (Preuss et al., 1997), and gamma irradiation (20–30 kGy) (Miekka et al., 1998).

THE PESTIVIRUS GENOME

The pestivirus genome, in the absence of insertions, is approximately 12.3 Kb in length (Collett et al., 1988a, b; Moormann et al., 1990; Deng and Brock, 1992; Ridpath and Bolin, 1995b, 1997). The long open reading frame (approximately 4000 codons) is bracketed by relatively large 5' (360–390 bases) and 3' (200–240 bases) untranslated regions (UTR). The 5' terminus does not contain a cap structure (Brock et al., 1992; Deng and Brock, 1993), and no poly(A) tract is present at the 3' end. All pestivirus genomes terminate at the 3' end with a short poly(C) tract. The highest nucleic acid sequence identity among pestiviruses is found in the 5' UTR (Ridpath and Bolin,

	10	20	30	40	50	60	70	80	90	100
BVDV1-SD-1	GTATACGAGA	ACTAGATAAA	ATACTCGTAT	ACA-TATTGG	ACAACAGAAA	ATAACTATTA	GGCC-TAGGG	AATGAATCCC	TCTCAGCGAA	GGCCGAAAGG
BVVF2-890	GTATACGAGA	-TTAGCTAAA	GTACTCGTAT	ACG-GATTGG	ACGTCGACAA	ACTTTGAATT	GGCAACACAG	GGAACCTTCC	CCTCGGCGAA	GGCCGAAAGG
CSFV-Alfort	C-----G	-TTAGCTCT-	-TTCTCGTAT	ACGATATTGG	ATA-CACTAA	ATTTTCGATT	GGTC-TAGGG	CA-----CCC	CTCCAGCGAC	GGCCGAAATG
BDV-BD31	G-----A	ACTACATCA-	-TACTCGTGT	ACAAAATTGG	ACA-TTCCAA	AACTCGATTG	GGT--TAGGG	AG-----CCC	TCTAGCGAC	GGCCGAAACG
	110	120	130	140	150	160	170	180	190	200
BVDV1-SD-1	AGGCTAGCCA	TGCCCTTAGT	AGGACTAGCA	TAATGAGGGG	GGTAGCAACA	GTGGTGAGTT	CGTTGGATGG	CTTAAGCCCT	GAGTACAGGG	TAGTCGTCTAG
BVVF2-890	AGGCTAGCCA	TGCCCTTAGT	AGGACTAGCC	AAAGGAGGGG	ACTAGCGGTA	GCAGTGAGTC	CATTGGATGG	CGGAACCCCT	GAGTACAGGG	GAGTCGTCTAA
CSFV-Alfort	-GGCTAGCCA	TGCCCATAGT	AGGACTAGCA	-AAGCGGAGG	ACTAGCGGTA	GTGGCGAGCT	CCCTGGGTGG	TCTAAGTCCT	GAGTACAGGA	CAGTCGTCTAG
BDV-BD31	-TGTTAGCCA	TACACGTAGT	AGGACTAGCA	-GACGGGAGG	ACTAGCCATG	GTGGTGAGAT	CCCTGAGCAG	TCTAAGTCCT	GAGTACAGGA	CAGTCGTCTAG
	210	220	230	240	250	260	270	280	290	300
BVDV1-SD-1	TGGTTCGAG	CCT-C---GG	TATAAAGGTC	TCBAGATGCC	ACGTGGACGA	GGGCACGCC	AAAGCACATC	TTAACCTGAG	CGGGGGTTCG	CCAGGCAAAA
BVVF2-890	TGGTTCGAG	CTC-CTTTAG	TCGAGGAGTC	TCBAGATGCC	ATGTGGACGA	GGGCATGCC	ACGGCACATC	TTAACCCACG	CGGGGGTTCG	GTGGGTGAAA
CSFV-Alfort	TAGTTCGAG	TGAGCACTAG	CCAC-----C	TCBAGATGCT	ACGTGGACGA	GGGCATGCC	AAGACACACC	TTAACCTGAG	CGGGGGTTCG	TAGGTGAAA
BDV-BD31	TAGTTCGAG	CAACAT--G	CTCTG----C	CTBAAATGCT	ACGTGGACGA	GG-CATGCC	AAGAC-TGC-	TTAATCTCGG	CGGGG-TCGC	CGAGGTGAAA
	310	320	330	340	350	360	370	380	390	400
BVDV1-SD-1	GCAGATCGAC	CAATCTGTGA	CGAATACAGC	CTGATAGGGT	GCTGCAGAGG	CCCACGTGAT	TGCTACTA--	AAAATCTCTG	CTGTACATGG	CACATGGAGT
BVVF2-890	GC--GCCATT	CGTGGCGTGA	TGGACACAGC	CTGATAGGGT	GCTGCAGAGG	CCTGCTACTC	CGCTAGTA--	AAAA-CTCTG	CTGTACATGG	CACATGGAGT
CSFV-Alfort	-----TCACA	TTATGTGATG	GGGGTACAGC	CTGATAGGGT	GCTGCAGAGG	CCCACGTGCA	GGCTAGTATA	AAAATCTCTG	CTGTACATGG	CACATGGAGT
BDV-BD31	-----CACC	TACCGGTGTT	GGGTACAGC	CTGATAGGGC	GCTGCAG--GG	CCCACGTATA	GGCTAGTATA	AAAATCTCTG	CTGTACATGG	CACATGGAGT

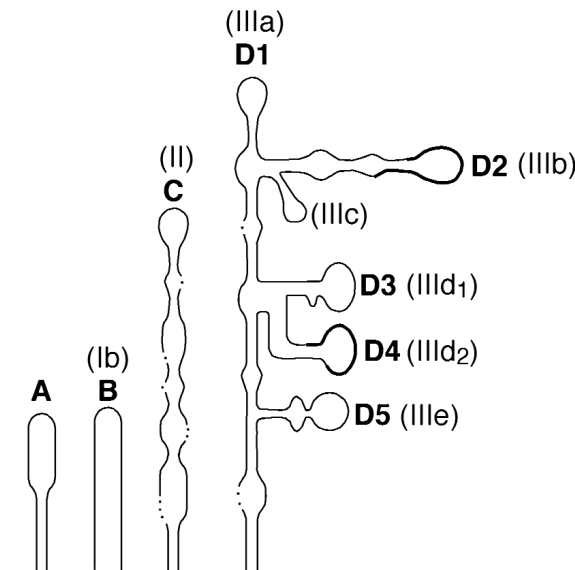


Figure 3.1. Conservation and predicted pseudoknots in pestivirus 5' UTR sequences. **Top.** The alignment and comparison of 5'UTR sequences from recognized pestivirus species is shown. Hypervariable regions are boxed. Conserved sequences used in RT-PCR tests to detect pestiviruses are underlined. The first ATG of the open reading frame is in bold type. **Bottom.** A schematic of predicted tertiary structures in the 5' UTR is shown. Deng and Brock (1993) domain designations are shown in bold, and Pestova and Hellen (1999) domain designations are shown in parentheses. Note that domain D5 of Deng and Brock is just a portion of the domain IIIf of Pestova and Hellen. The location of hypervariable regions shown in Figure 3.1a are denoted by a thickened line in the schematic.

1997). Although sequence identity is high among pestiviruses in the 5' UTR, there are two short regions that are notable for their variability (Figure 3.1A). These are located between nucleotides 208–223 and nucleotides 294–323 (nucleotide position numbers based on the sequence of BVDV 1-SD-1¹). Sequence variations in these regions have been ex-

ploited in PCR-based tests designed to differentiate BVDV genotypes (el-Kholy et al., 1998; Ridpath and Bolin, 1998). High conservation of 5' UTR sequences is related to formation of tertiary structures required for internal ribosomal entry mediated initiation of translation (Deng and Brock, 1993; Pestova and Hellen, 1999) (Figure 3.1B).

¹Although BVDV 1a-NADL and BVDV 2-890 are the type viruses for genotypes BVDV 1 and BVDV 2, respectively, both genomes have inserted sequences. Insertions can cause confusion when indicating genomic location based on nucleotide number. For this reason BVDV 1a-SD-1 is used as the reference for nucleotide position. It was the first noncytopathic BVDV1 sequenced and does not have an insertion. The accession number for BVDV 1a-SD-1 is M96751.

ENTRY OF THE VIRUS INTO CELLS, TRANSLATION, AND REPLICATION

The binding and entry of BVDV to susceptible cells has not been extensively studied. Based on the behavior of other flaviviruses and pestiviruses, it is hypothesized that binding and entry is a multistep process initiated by receptor-mediated endocytosis involving cell surface molecules and the viral proteins E^{rns} and E2 (Schelp et al., 1995; Xue et al., 1997; Iqbal et al., 2000; Schelp et al., 2000; Iqbal and McCauley, 2002). The genomic RNA is uncoated following endocytosis and serves as the mRNA. No subgenomic mRNA molecules have been detected. Initiation of translation is mediated by a cap-independent internal initiation mechanism that requires an internal ribosome entry site (IRES) located within the 5' UTR. A secondary structure model for the pestivirus 5' UTR was first proposed by Deng and Brock (1993). Further refinements of a BVDV specific model have been developed (Poole et al., 1995; Pestova et al., 1998; Pestova and Hellen, 1999). Unfortunately a universal terminology system was not adopted. The different names used for the same structural domains by different research groups can be confusing. The proposed 5' UTR secondary structures shown in Figure 3.1B are identified by both the domain names given by Deng and Brock and those given by Pestova and Hellen. It should be noted that domain D5 of Deng and Brock is just a portion of the IIIe domain of Pestova and Hellen. The IIIe domain contains the D5 domain plus the nucleotides between D5 and nucleotide 380 (numbering based on BVDV 1a-SD-1). Domains C (II) and D (III) contain structures critical for the attachment of the initiation complex. A pseudoknot formed just upstream of the start codon is necessary for IRES function. The hypervariable regions used in PCR based tests to differentiate BVDV genotypes are located in domains D2 (IIIb) and D4 (IIIId2).

VIRAL PROTEINS

The large open reading frame is translated as a polyprotein. The order of the individual viral proteins within the polyprotein is as follows: N^{pro} -C- E^{rns} -E1-E2-p7-NS2/3-NS4A-NS4B-NS5A-NS5B (Figure 3.2A). The polyprotein is processed cotranslationally and posttranslationally by host and viral proteases. The size, function, and characteristics of the viral proteins are summarized in Table 3.1. Conservation of the polyprotein coding sequences among pestiviruses is shown in Figure 3.2B. The predicted hydrophobicity profile of the

polyprotein is shown in Figure 3.2C. The hydrophobicity profile of the polyprotein is conserved within the pestivirus genus.

Viral structural proteins

The proteins associated with the mature virion are C, E^{rns} , E1, and E2. C is the virion nucleocapsid protein. It is highly basic and relatively conserved among the pestivirus species. It is postulated that the C terminus of the nucleocapsid protein contains an internal signal sequence that directs translocation of structural glycoproteins to the endoplasmic reticulum (Heinz et al., 2000). The E^{rns} protein is glycosylated and forms disulfide-linked homodimers (Weiland et al., 1990). In tissue culture systems E^{rns} may be found both associated with released virus and free in the culture medium. This protein has an unusual ribonuclease activity. Although the function of this ribonuclease activity in the viral life cycle is unknown, antibodies that inhibit ribonuclease activity neutralize virus infectivity of classical swine fever viruses (CSFV) (Windisch et al., 1996). The ribonuclease activity does not require dimer formation or glycosylation (Windisch et al., 1996). The E^{rns} of CSFV possesses epitopes that induce neutralizing antibodies (hereafter referred to as neutralizing epitopes) and CSFV E^{rns} subunit vaccines induce neutralizing antibodies (Konig et al., 1995). It is not known whether the E^{rns} of BVDV possesses neutralizing epitopes that are important in disease control. However, protection afforded by killed BVDV vaccines does not appear to be dependent upon E^{rns} specific antibodies (Bolin and Ridpath, 1990).

Both the E1 and E2 proteins possess potential membrane-spanning domains and are glycosylated. They are predicted to be integral membrane proteins and interact to form heterodimers (Weiland et al., 1990). The E2 protein is the immunodominant structural protein and possesses neutralizing epitopes (Donis et al., 1988; Bolin and Ridpath, 1989). Mabs produced against the E2 have been used to differentiate pestivirus species (Peters et al., 1986; Wensvoort et al., 1986; Bolin et al., 1988; Hess et al., 1988; Wensvoort et al., 1989a; Corapi et al., 1990a; Edwards and Sands, 1990; Dahle et al., 1991; Deregt et al., 1994; Paton et al., 1994; Ridpath et al., 1994a; Ridpath and Bolin, 1997). The coding region for E2 neutralizing epitopes are found in a hypervariable region located in the N-terminal portion of the E2 protein (Paton et al., 1992; Deregt et al., 1998) (refer to Figure 3.2B). E2 antigenic variation is particularly pronounced among BVDV strains and contributes to vaccine failure (Bolin et al., 1988; Bolin and Rid-

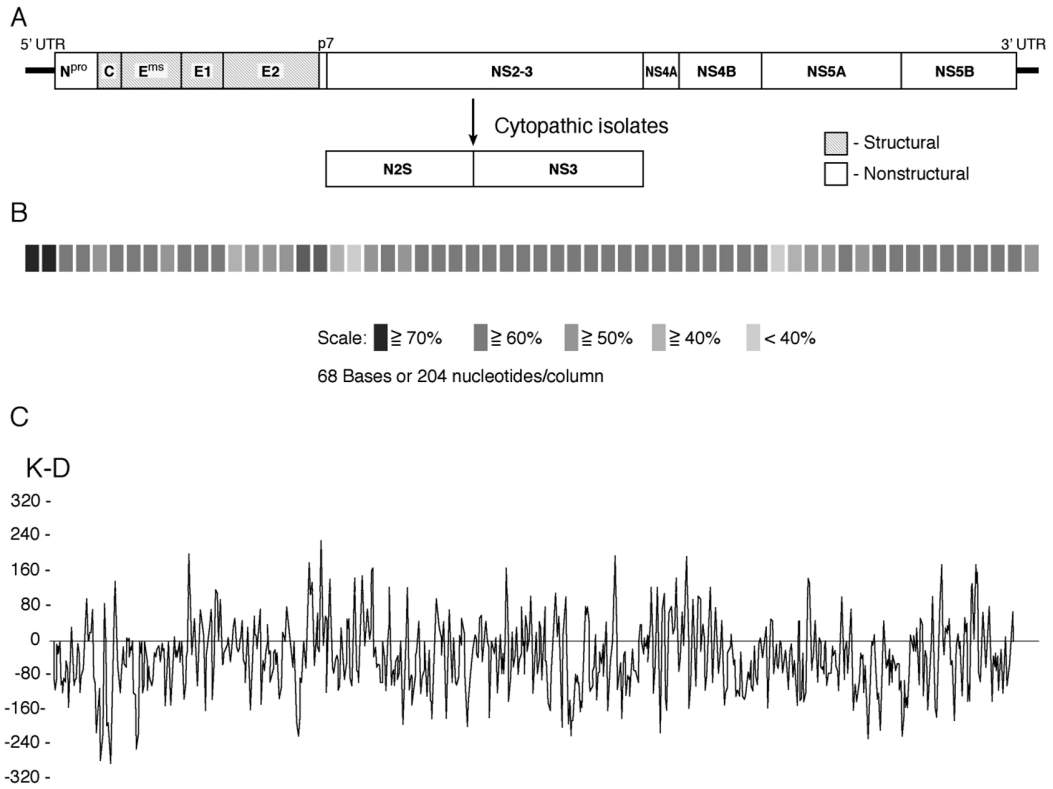


Figure 3.2. The pestivirus genome. **A.** Organization of the pestivirus genome is shown. Structural proteins are denoted by shading. **B.** Sequence conservation among pestivirus species is shown. These results are a composite of comparisons between the four recognized pestivirus species. Sequence similarity is indicated by different levels of shading. **C.** A hydrophobicity plot of a pestivirus consensus sequence is shown. A consensus sequence was derived by comparison of representatives of noncytopathic strains from each of the four recognized pestivirus species.

path, 1989, 1990; Bolin et al., 1991a; Bolin et al., 1994; Ridpath et al., 1994a; Van Campen et al., 1997; Bolin and Ridpath, 1998; Van Campen et al., 1998; Ridpath et al., 2000; Van Campen et al., 2000).

Viral nonstructural proteins

The first viral protein encoded by the BVDV ORF is the nonstructural protein, N^{pro}. This protein, which is unique to the pestivirus genus, is an autoprotease whose only known function is to cleave itself from the polyprotein. The next nonstructural protein, p7, follows the structural protein E2 in the polyprotein (Elbers et al., 1996). It consists of a central charged region flanked by hydrophobic termini. The role of this cell-associated protein is unknown. It is hypothesized that p7 is required for production of infectious virus but not for RNA replication (Harada et al., 2000). It is inefficiently cleaved from the E2, leading to two intracellular forms of E2 with different C termini (E2 and E2-p7) (Elbers et al., 1996).

Neither p7 nor E2-p7 are found associated with infectious virus.

Following p7 the next nonstructural protein in the polyprotein is the serine protease, NS2-3. In BVDV strains from the cytopathic biotype (see discussion of biotype below), the NS2-3 is cleaved to NS2 and NS3. The serine protease activity of the NS2-3 resides in the NS3 portion of the protein. The function of the NS2 is unknown. It is not required for RNA replication, and its cleavage from the NS2-3 does not affect serine protease activity (Behrens et al., 1998). Sequence analysis reveals regions in the NS2 with homology to zinc-finger motifs present in DNA binding proteins (De Moerloozee et al., 1991). However, a function for the putative DNA binding activity of NS2 in viral replication has not been demonstrated.

Both the uncleaved NS2-3 and the cleaved NS3 act as serine proteases (Tautz et al., 1997) that cleave the remaining nonstructural proteins from the

Table 3.1. Pestivirus proteins

Viral Protein	Estimated Size (K Daltons)	Attributes	Neutralizing Epitope(s)	Function
N ^{pro}	20	Nonstructural	N	Autoproteolysis Not required for RNA replication
C	14	Structural Conserved Highly basic	N	Forms nucleocapsid of virion
E ^{rns}	48	Structural 7–9 glycosylation sites	Y	Envelope-associated glycoprotein Ribonuclease activity
E1	25	Structural 2–3 glycosylation sites	N	Envelope-associated glycoprotein Integral membrane protein
E2	53	Structural 4–6 glycosylation sites	Y	Envelope-associated glycoprotein Integral membrane protein
p7	7	Least conserved of structural proteins Nonstructural Central charged region flanked by hydrophobic termini	N	Immunodominant structural protein Function unknown Required for production of infectious virus but not RNA replication
NS 2/3	125	Nonstructural	N	NS2 has a zinc-finger-like domain
NS2	54	In cytopathic biotype NS 2/3 cleaved to NS2 and NS3		NS2/3 and NS3 contain RNA helicase and N-terminal serine protease domains; cleaves itself and remaining nonstructural proteins from viral polyprotein
NS3	80	Conserved		Immunodominant nonstructural protein
NS4A	7.2	Nonstructural Hydrophobic	N	Serine protease cofactor
NS4B	38–39	Nonstructural Hydrophobic	N	Replicase component
NS5A	55–56	Nonstructural Phosphorylated	N	Replicase component
NS5B	81–82	Nonstructural	N	RNA-dependent RNA polymerase

polyprotein. Purified BVDV NS3 also possesses RNA helicase and RNA-stimulated NTPase activities (Tamura et al., 1993; Warrenner and Collett, 1995). All three activities (serine protease, RNA helicase, and RNA-stimulated NTPase) are essential to virus viability (Grassmann et al., 1999; Gu et al., 2000). Antibodies to the NS2-3 and NS3 do not neutralize infectivity. However, the NS2-3 and NS3 (but not the NS2) are strongly recognized by polyclonal convalescent sera (Donis et al., 1991). Animals vaccinated with modified live vaccines also have a strong antibody response to the NS2-3 and/or NS3 protein (Bolin and Ridpath, 1989). In contrast, animals vaccinated with inactivated (killed) vaccines

primarily react with structural proteins and not the NS2-3 or NS3 (Bolin and Ridpath, 1990). The difference in recognition of NS2-3 or NS3 may be useful in differentiating between immune responses to inactivated vaccines and immune responses to natural infection.

The NS4A and NS4B proteins are similar in size, composition and hydrophobicity to the NS4A and NS4B proteins of other flaviviruses (Lindenbach and Rice, 2001). NS4A acts as a cofactor for the NS2-3 and NS3 serine protease activity. NS4B and NS5A probably are replicase complex components. RNA polymerase activity has been demonstrated for the NS5B protein (Lai et al., 1999).

Nomenclature for proteins

The names of the viral proteins have changed over time and these variations in terminology can be confusing as one reads past literature. The names used above and in Table 3.1 are those designated in the Seventh Report of the International Committee on Taxonomy of Viruses (van Regenmortel et al., 2000). The first studies of BVDV reported two glycosylated structural polypeptides, with molecular weights of 55K and 45K, which were termed VP1 and VP2, respectively (Matthaeus, 1979). Later reviews referred to these proteins as E1 and E2 (Westaway et al., 1985). When Collett et al. first derived the genomic map for BVDV, gene products were designated by their molecular weights and glycosylation status (Collett et al., 1988b). Thus, the viral protein now known as E2, may be referred to as VP1, E1, or gp53 in the literature. Similarly, E^{ns} may be reported as VP2, E0, E2, or gp48.

TRANSLATION AND PROCESSING OF THE POLYPROTEIN

As stated above, the first viral protein in the ORF is a nonstructural protein unique to the pestivirus genus, N^{pro}. The N^{pro} is an autoprotease whose only known function is to cleave itself from the polyprotein. Cellular signal peptidases are responsible for the cleavages between C and E^{ns}, E1, and E2, and E2 and p7 (Rumenapf et al., 1993; Stark et al., 1993; Elbers et al., 1996). The mechanism that produces the cleavage between E^{ns} and E2 is unknown. As stated above, the cleavage between E2 and p7 may not be complete. Depending on the biotype of the virus (see section on biotypes below), the NS2/3 protein may be further processed to the NS2 and the NS3 proteins. Both NS2/3 and NS3 have serine protease activity that cleaves the remaining downstream proteins from the polyprotein. The NS4A viral protein facilitates the protease activity of the NS2/3 (and/or NS3) at the cleavage between NS4B and NS5A and between NS5A and NS5B.

VIRAL REPLICATION

Viral proteins, generated by processing the translated polyprotein, participate in viral replication. It has been proposed that a secondary structure motif in the 5' UTR enables the switch of viral RNA from a template for translation to a template for replication (Yu et al., 2000). RNA replication occurs via a replication complex, composed of viral RNA and viral nonstructural proteins, in association with intracytoplasmic membranes. The replication of BVDV RNA is similar to that of other flaviviruses.

After translation, RNA replication begins with the synthesis of complementary negative strands. Using these negative strands as templates, genome-length positive strands are synthesized by a semiconservative mechanism involving replicative intermediates and replicative forms (Gong et al., 1996; Gong et al., 1998). Because viral proteins are not detected on the surface of infected cells, it is thought that virions mature in intracellular vesicles and are released by exocytosis (Heinz et al., 2000). A substantial fraction of the infectious virus remains cell associated (Lindenbach and Rice, 2001).

BVDV GENOTYPES AND PHENOTYPES

As stated above, BVDV strains may belong to one of two different genotypes, BVDV 1 and BVDV 2. Further, viruses from either genotype may exist as one of two biotypes, cytopathic and noncytopathic.

BVDV GENOTYPES

Genotyping is grouping based on comparison of genomic sequences. There are no hard and fast taxonomic rules governing how different two genomes have to be before they are designated different genotypes. However, for genotypic designations to be meaningful, some practical considerations should be taken into account. Genotypes should be distinct, discrete, and stable. That is, there should be a clear defined break between groups (discrete groups as opposed to a continuum), and isolates should not be able to easily switch back and forth between groups. Ideally genotypes would be associated with practical observations such as geographic distribution, antigenic variation, or variations in virulence. The differentiation between the BVDV 1 and the BVDV 2 genotypes meets these practical considerations.

The first reported division of BVDV strains into two different genotypes was based on comparison of the 5' UTR (Pellerin et al., 1994; Ridpath et al., 1994a). Previous studies of vaccination cross protection and monoclonal antibody binding revealed that BVDV strains were quite variable (Moennig et al., 1987; Bolin et al., 1988; Corapi et al., 1990a; Bolin et al., 1991a; Donis et al., 1991; Edwards et al., 1991). Although these studies indicated that there was considerable variation among BVDV strains, no standard means of grouping viruses based on these variations was generated. Meanwhile, hybridization analysis and sequence comparison suggested that the 5' UTR was highly conserved compared to other portions of the genome (Lewis et al., 1991; Ridpath and Bolin, 1991; Ridpath et al.,

1993). The highly conserved sequences in the 5' UTR were evaluated by several groups as targets for polymerase chain reaction (PCR) tests designed to detect the wide range of BVDV strains or to differentiate BVDV from CSFV or BDV (Hofmann et al., 1994; Vilcek, 1994). Concurrent to this research a highly virulent form of BVD, termed hemorrhagic syndrome, was reported in Canada and the United States (Corapi et al., 1989; Corapi et al., 1990b; Carman, 1995; Carman et al., 1998). Phylogenetic analysis of the BVDV strains, isolated from animals suffering from hemorrhagic syndrome, grouped them separately from the BVDV strains commonly used, at that time, in vaccine production, diagnostic tests, and research (Pellerin et al., 1994; Ridpath et al., 1994a). The newly recognized group of BVDV was designated BVDV genotype II, and the group containing the strains used in vaccines, detection, and research was termed BVDV genotype I. The names of these two genotypes were later modified to BVDV 1 and BVDV 2 in keeping with taxonomic conventions in use with other viruses (Heinz et al., 2000). It was further noted that viruses from the BVDV 2 genotype were also isolated from persistently infected calves born to dams that had been vaccinated with vaccines based on BVDV 1 isolates (Ridpath et al., 1994a). Comparison of monoclonal antibody binding profiles revealed clear differences between BVDV 1 and BVDV 2 strains (Ridpath et al., 1994a). Although initial phylogenetic differentiation was based on comparison of 5' UTR sequences, comparison of complete genomic sequences showed that differences between BVDV 1 and BVDV 2 strains were found throughout the genome (Ridpath and Bolin, 1995b).

There are two common misconceptions relating to the BVDV 2 genotype. One relates to origin of the BVDV 2 genotype and the other relates to the virulence of BVDV 2 strains. In the late 1990s there was some speculation that BVDV 2 strains represented newly emerging viruses that originated in the United States as a result of use of vaccines by U.S. producers and were then transferred to Europe. Although BVDV 2 strains were first recognized in 1994, retrospective characterization of strains collected from BVDV outbreaks in Ontario that occurred between 1981 and 1994 demonstrated that BVDV 2 were present in North America at least since the early 1980s. However, the first BVDV 2 strain described in the literature was isolated in Europe prior to 1979 (Wensvoort et al., 1989b). Interestingly, this strain was isolated from a pig and was referred to as an atypical CSFV. Subsequent comparison of this iso-

late to BVDV 2 strains isolated from North America led to the segregation of this virus to the BVDV 2 genotype (Paton et al., 1995b). Further, a phylogenetic survey of BVDV 1 and BVDV 2 strains showed similar levels of sequence variation in the 5' UTR (Ridpath et al., 2000). If one assumes that the evolutionary clock ticks at the same rate for both genotypes, similar rates of variation in the 5' UTR suggests that the two genotypes have been evolving for approximately the same time span. This suggests that viruses from the BVDV 2 genotype are not newly emerging viruses, but newly recognized ones.

Initially the BVDV 2 genotype was associated with outbreaks of hemorrhagic syndrome (Corapi et al., 1989; Corapi et al., 1990b; Pellerin et al., 1994; Carman et al., 1998), a clinically severe form of acute BVDV infection. This led to the misconception that all BVDV 2 strains cause severe acute disease. In an initial survey of BVDV 2 strains, only 32 out of 76 strains were associated with clinically severe disease (Ridpath et al., 1994a). Since then, animal studies have shown that there is a continuum of virulence seen in acute infections with type 2 BVDV (Flores et al., 2000; Fulton et al., 2000a; Hamers et al., 2000; Ridpath et al., 2000; Tajima et al., 2001; Evermann and Ridpath, 2002; Kelling et al., 2002; Liebler-Tenorio et al., 2002; Liebler-Tenorio et al., 2003). Thus, it appears that only a minority of BVDV 2 strains cause severe acute clinical disease. The majority of BVDV 2 strains present in North America appear to be no more virulent than BVDV 1 strains.

Prevalence of BVDV genotypes

Both BVDV genotypes have been reported in North America (Pellerin et al., 1994; Ridpath and Neill, 1998), Europe (Becher et al., 1995; Edwards and Paton, 1995; Paton, 1995a, b; Sandvik et al., 1997; van Rijn et al., 1997; Giangaspero et al., 2001; Luzzago et al., 2001; Pratelli et al., 2001; Couvreur et al., 2002; Vilcek et al., 2002), and South America (Canal et al., 1998; Jones et al., 2001), although the reported prevalence of BVDV 2 is higher in North America than in Europe or South America. Reports of the prevalence of the BVDV 2 genotype in commercial cattle herds in North America range from 24–47% (Carman, 1995; Bolin and Ridpath, 1998; Carman et al., 1998; Fulton et al., 1998; Fulton et al., 2000b; Evermann and Ridpath, 2002).

Similarities between viruses from the BVDV 1 and the BVDV 2 genotype

Viruses from both genotypes may exist as one of two biotypes, cytopathic and noncytopathic (see

below). Noncytopathic viruses from either genotype can cross the placenta and establish persistent infections in the fetus.

Aside from severe acute BVDV, it is difficult to distinguish BVDV 1 infections from BVDV 2 infections based on clinical signs. There is considerable heterogeneity among strains from both genotypes. Although virulence is strain-dependent, clinical presentation may also be effected by immune status, reproductive status, stress, and the presence of secondary pathogens.

Heterogeneity, among strains within each genotype, also affects antigenicity. Although strains from the BVDV 1 and BVDV 2 genotypes may be distinguished by monoclonal antibody binding, there are smaller, but still significant, differences among strains from the same genotype (Ridpath et al., 1994a; Ridpath et al., 2000).

Differences between viruses from the BVDV 1 and the BVDV 2 genotype

To date only viruses from the BVDV 2 genotype are associated with severe acute BVDV (Corapi et al., 1989; Corapi et al., 1990b; Bolin and Ridpath, 1992; Pellerin et al., 1994; Ridpath et al., 1994a; Carman et al., 1998; Liebler-Tenorio et al., 2002). BVDV 1 and BVDV 2 strains are antigenically distinct, as demonstrated by serum neutralization using polyclonal sera and monoclonal antibody binding (Ridpath et al., 2000). The practical significance of antigenic differences is indicated by the birth of BVDV 2 persistently infected animals to dams that had been vaccinated against BVDV 1 strains (Bolin et al., 1991a; Ridpath et al., 1994a). Although modified live BVDV 1 vaccines may induce antibodies against BVDV 2 strains, the titers average one log less than titers against heterologous BVDV 1 strains (Cortese et al., 1998).

Subgenotypes of BVDV 1 and BVDV 2

Subgenotypes of both BVDV 1 and BVDV 2 have been described, although the biological significance of these subgroupings is a matter of debate. BVDV 1 strains from North America can be segregated to two subgenotypes, BVDV 1a and BVDV 1b (Pellerin et al., 1994). These two subgenotypes can be distinguished by monoclonal antibody binding (Bolin and Ridpath, 1998) and RT-PCR (Ridpath and Bolin, 1998). Epidemiological surveys suggest that BVDV 1b strains may predominate in respiratory cases (Fulton et al., 2002), and BVDV 1a strains may predominate in fetal infections occurring late in gestation (>100 days gestation) (Evermann and Rid-

path, 2002). While the reported incidence of BVDV 2 strains is lower, European BVDV 1 strains appear to be more variable than North American BVDV 1 strains. The European BVDV 1 strains have been separated into at least 11 subgroups (Vilcek et al., 2001a). The biological significance of these 11 groups has yet to be examined.

Similarly, BVDV 2 strains from North and South America have been segregated into two subgroups, BVDV 2a and BVDV 2b (Flores et al., 2002). In North America BVDV 2b strains are relatively rare; in South America the prevalence of BVDV 2a strains and BVDV 2b strains are similar.

BVDV BIOTYPES

As stated above, both BVDV 1 strains and BVDV 2 strains may exist as one of two biotypes, cytopathic and noncytopathic. The division into biotypes is based on the activity of the strain when propagated in cultured epithelial cells (Lee and Gillespie, 1957; Gillespie et al., 1960). Recall that both noncytopathic and cytopathic strains code for the nonstructural protein NS2-3. Among cytopathic strains the NS2-3 is processed to an NS2 and an NS3 protein. Processing of the NS2-3 protein in cytopathic strains may occur by several different strategies depending on the viral strain (Meyers et al., 1991a, b; Meyers et al., 1992; Qi et al., 1992; Ridpath et al., 1994b; Kupfermann et al., 1996; Tautz et al., 1996; Becher et al., 1998; Mendez et al., 1998; Meyers et al., 1998; Qi et al., 1998; Becher et al., 1999; Baroth et al., 2000; Kummerer and Meyers, 2000; Ridpath and Neill, 2000; Vilcek et al., 2000; Neill and Ridpath, 2001; Becher et al., 2002; Ridpath and Bolin, 1995a). Most commonly, the generation of the NS3 is associated with insertion of sequences into the viral genome. Generally the amino acid sequence flanking the carboxy-terminus of the insertion corresponds to either amino acid position 1535 or position 1589 (numbering based on the noncytopathic BVDV 1a strain SD-1, acc. # M96751, see footnote a). These two positions are referred to as positions A and B, respectively (Meyers et al., 1998). Position B corresponds to the cleavage site that frees the N-terminus of the NS3 from the NS2-3 precursor. It is thought that insertions at the B position result in the processing of the NS2-3, either by introducing a new cleavage site at carboxy terminus of the insertion or by introducing sequences with autocatalytic activity that act at the carboxy terminus of the insertion. The end result is that cleavage occurs at position 1589, making Gly₁₅₈₉ the first amino acid of NS3. Insertions at position A, al-

though upstream of the cleavage site, also produce cleavage at amino acid position 1589. It has been proposed that insertions upstream of the cleavage site induce conformational changes that allow cleavage via a cryptic mechanism at Gly₁₅₈₉ (Meyers and Thiel, 1996). The most frequently described insertions into cytopathic BVDV 1 strains have been at position B and consist of duplicated viral genomic sequence and/or ubiquitin coding sequences. Insertions in cytopathic BVDV 2 strains are more frequently reported in the vicinity of position A and consist of portions of a gene coding for a DnaJ-like protein (Ridpath and Neill, 2000; Neill and Ridpath, 2001). Included among the possible explanations for these differences in relative frequency of position and source of inserted sequences are (i) differences in recombination frequencies due to sequence variation; (ii) differences in the stability of recombinations; and (iii) differences in the relative amounts of mRNA coding for ubiquitin and DnaJ-like proteins in BVDV 1 and BVDV 2 infected cells.

Practical significance of biotypes

Only noncytopathic BVDV have been reported to establish persistent infections. This has led to some speculation that cytopathic BVDV are not able to cross the placenta and thus do not infect the fetus. However, the detection of cytopathic BVDV strains in fetal calf serum and fetal sero conversion in utero after exposure to cytopathic BVDV call into question this assumption (Brownlie et al., 1989; Bolin et al., 1991b; Bolin and Ridpath, 1998). Because cytopathic BVDV do not establish persistent infections in vivo, they are frequently the biotype of choice for modified live vaccines. Superinfection of an animal, persistently infected with a noncytopathic BVDV, with a cytopathic BVDV can lead to the development of a highly fatal form of BVDV called mucosal disease (Brownlie et al., 1984; Bolin et al., 1985). Mucosal disease is a comparatively rare form of BVDV and does not represent a major source of loss for producers (Houe, 1995, 1999). The majority of losses incurred by producers due to BVDV infections are associated with acute infections with noncytopathic BVDV (Houe, 1995; Fulton et al., 1998; Houe, 1999; Fulton et al., 2000a, b; Evermann and Ridpath, 2002; Fulton et al., 2002).

Noncytopathic BVDV strains predominate over cytopathic BVDV in nature. In the field, cytopathic BVDV strains are typically isolated from mucosal disease outbreaks or post-vaccinal disease outbreaks. Cytopathology in cultured epithelial cells does not correlate with virulence in acute infections

in vivo. All highly virulent viruses studied to date have been noncytopathic (Corapi et al., 1990b; Bolin and Ridpath, 1992; Carman et al., 1998; Ellis et al., 1998; Ridpath et al., 2000; Liebler-Tenorio et al., 2002). In short, from a management standpoint, determining the genotype of a strain is generally more important than determining the biotype.

Importance of variation among BVDV

Heterogeneity is the defining characteristic of BVDV viruses. Variation between BVDV genotype 1 and BVDV genotype 2 has practical significance. In particular, variation in antigenicity impacts on detection and management programs. The impact of variation within each genotype is less well defined. Understanding and compensating for variation among BVDV is essential to developing a successful control program.

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4

Virus Replication

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INTRODUCTION

RNA viruses, including bovine viral diarrhea virus (BVDV), replicate and survive through diverse genetic strategies and lifecycles that rely on the biology and biochemistry of their hosts. Like other viruses, infection of host cells by BVDV results in modifications in gene expression that would be beneficial to virus replication and survival. Up-regulation of genes encoding host proteins involved in translation and posttranslational processing, and nonlethal down-regulation of genes encoding proteins involved in energy production and cell structure have been documented in cells infected with BVDV (Neill and Ridpath, 2003a, 2003b). As seen with other viruses possessing both cytopathic and noncytopathic biotypes, cytopathic BVDV amplifies viral RNA at levels that are several logs higher when compared to their noncytopathic counterparts (Glew et al., 2003; Kummerer and Meyers, 2000; Mendez et al., 1998; Vassilev and Donis, 2000).

A semiconservative asymmetric model has been used to describe BVDV RNA replication. Based on finding of negative-strand template and nascent positive-strand RNA, BVDV replication has been detected in cell culture models within 4–6 hours of infection, with peak BVDV titer detected at 12–24 hours postinfection (Gong et al., 1996; Purchio et al., 1983).

BVDV replication occurs through an RNA-dependent RNA synthesis pathway that utilizes enzyme activities not typically found in uninfected host cells, and therefore, must be encoded in the viral genome and expressed during infection. In eukaryotic cells, ribosomes require a specific methylated cap structure at the 5' end of the genome to signal the initiation of protein synthesis, and in general, each mRNA results in a single polypeptide product. As a family, the RNA viruses have evolved unique

mechanisms to obtain multiple protein products from a single genome by fragmentation at the level of protein, mRNA, or the gene. Pestiviruses, including BVDV, utilize their single-stranded positive-sense genome as template for both translation and replication. BVDV relies on fragmentation by extensive cotranslation and posttranslation proteolytic processing using host and viral proteases to obtain at least 11 viral proteins from a single translated polyprotein.

VIRAL GENOME

GENOME ORGANIZATION

The BVDV genome consists of single-stranded positive-sense RNA approximately 12.5 kb in length, with some variability in size associated with genomic deletions, insertions, and duplications. Untranslated regions at the 5' and 3' ends of the genome (5' UTR and 3' UTR, respectively) flank a large open reading frame (ORF) that encodes the approximately 4,000 amino acid polyprotein. The high degree of conservation of nucleotide sequence and structure for BVDV and the other pestiviruses in the 5' and 3' regions of the genome indicates specific genomic elements required for positive-strand synthesis, translation, and possibly packaging of viral genome (Becher et al., 1999b; Becher et al., 2000; Chon et al., 1998; Deng and Brock, 1993; Yu et al., 1999).

The organization of the BVDV genome is N^{pro}, Capsid, E^{ns}, E1, E2, and p7, which code for structural proteins, followed by NS2-3, NS4A, NS4B, NS5A, and NS5B encoding nonstructural proteins. The genomic organization in cytopathic strains of BVDV can deviate based on the variety of genomic insertions, deletions, and replications. The function and description of proteins encoded by the BVDV

genome are described in the preceding chapter. In brief, the viral-encoded proteins involved in BVDV replication include an autoprotease encoded by N^{pro}, NS3-encoded serine protease, ATPase, and RNA helicase, an NS4A-encoded essential cofactor of NS3 serine protease, an NS5A-encoded serine phosphoprotein, and the NS5B encoded RNA-dependent RNA polymerase. The function of nonstructural proteins NS4B and NS5A has not been identified, but these proteins have been associated with a multiprotein complex also involving NS3. Blockage of the ion channel encoded by the small structural protein p7 has recently been shown to suppress replication of BVDV (Pavlovic et al., 2003).

5' UTR

Translation among the pestiviruses is cap-independent, relying instead on an internal ribosomal entry site (IRES), which mediates internal attachment of ribosomes to the translation initiation codon. The BVDV IRES is encoded in the 5' UTR and contains essential structural or functional components that may extend into or include nucleotide sequence within the ORF. Although the exact genomic boundaries of the IRES have not yet been clearly defined, the structural integrity in the region, divided among three domains and including a pseudoknot structure, is required for IRES activity (Fields et al., 2001). Structural and biochemical models demonstrate that the two stem-loop structures located at the 5' of the genome and nucleotides of the N^{pro}-coding region are required for efficient RNA replication. Proper secondary structure in the stem of the 5' terminal stem-loop was found to be critical to efficient translation, and an intact loop and portion of the stem are important components for replication (Yu et al., 2000). Based on similarities with other *Flaviviridae* genomes, it is postulated that the pseudoknot structurally serves to position the ribosomal subunit over the initiator AUG codon (Lemon and Honda, 1997). The 5' UTR of BVDV, similar to other pestiviruses, is relatively long at approximately 385 nucleotides and contains multiple initiator codons upstream of the actual translation initiation site (Yu et al., 2000). Additionally, four initial nucleotides at the 5' terminus of the BVDV genome have been reported to provide an essential signal for replication (Frolov et al., 1998; Yu et al., 2000).

3' UTR

The 3' end of the BVDV genome also contains critical primary and secondary structures, including a conserved single-stranded region separating two

hairpin loops that functions to direct initiation of negative-strand synthesis and is critical for replication (Fields et al., 2001; Yu et al., 1999). The single-stranded region separating the two stem-loop structures is highly conserved among the pestiviruses (Becher et al., 1998b; Yu et al., 1999). It has been suggested, based on similarities to other single-stranded RNA viruses, that the structurally conserved 3' region may be involved in regulatory functions by cross-talk with the 5' structural components (Yu et al., 1999). If analogous to other RNA viruses, the structural interaction of the 5' and 3' regions could be involved in modulation of RNA-RNA interactions, translation, replication, and encapsulation steps.

VIRUS BINDING AND ENTRY

Based on comparisons to related members of the *Flaviviridae*, the binding and entry of BVDV involves a series of steps, beginning with attachment or interaction of the virion with specific host cell receptors, followed by internalization and pH dependent fusion of the viral envelope and cell membrane. Envelope glycoproteins coded by E2 and E^{ms} have been demonstrated to independently bind to cell surfaces (Hulst and Moorman, 1997; Iqbal et al., 2000). The ability of BVDV to infect a relatively diverse range of cell-types, as well as the tissue and host-species tropisms observed for BVDV have been associated with the E2 envelope glycoprotein (Liang et al., 2003). The E2 protein is translated from a highly variable region of the genome and may additionally contribute to the ability of BVDV to escape the host immune response (Ridpath, 2003). Though specific cell receptors for BVDV entry have not been well characterized, low-density lipoprotein receptors have been identified (Baranowski et al., 2001). Candidate receptors, as determined by monoclonal antibodies capable of blocking infection, include 50kDa, 60kDa, and 93kDa cell surface proteins found on bovine cells (Minocha et al., 1997; Schlep et al., 1995; Xue and Minocha, 1993; Xue et al., 1997). After entry into the host cell is complete, viral RNA is released into the host cell cytoplasm and RNA translation begins.

TRANSLATION AND REPLICATION

Initiation of the translation process is mediated by the IRES. It has been demonstrated among pestiviruses that the IRES binds specifically to the 40S ribosomal subunit in the absence of any additional translation initiation factors. In concert with cellular

components, and functionally linked to the translation process, viral nonstructural proteins assemble into a functional replicase complex to catalyze transcription of positive-sense RNA into full-length complementary strand negative-sense RNA. The negative RNA strands provide template for the replicase complex to synthesize additional positive-sense RNA molecules, using a semiconservative asymmetric replication model (Warrilow et al., 2000). The model includes three virus-specific RNAs: a double-stranded replicative form (RF), a partially single-stranded and partially double-stranded replicative intermediate (RI), and single-stranded viral RNA.

The replication process begins with a positive-strand replicase complex comprised of viral and cellular components formed at the 3' terminus of the genome. Progression from initiation to elongation occurs after the synthesis of nascent RNAs 8–10 nucleotides in length (Sun and Kao, 1997). Elongation mediated by viral-polymerase displaces the positive strand from the RI template, allowing recycling of the template while elongation of the prior nascent strand continues. Approximately six nascent positive strands per template have been predicted (Gong et al., 1998). The double-stranded replicative form is a product of viral RNA used as template for minus-strand synthesis, or may represent the replicative intermediate during synthesis of the final nascent strand (no new initiation complexes formed). During *in vitro* experiments, the ratio of positive-to-minus-sense RNA increases from 2:1 at 4 hours to 10:1 by 12 hours postinoculation (Gong et al., 1996).

GENETIC RECOMBINATION

The high rate of genetic insertion and recombination events in BVDV as compared to other positive-sense RNA viruses is unique to pestiviruses and is possibly related to some unique character of the pestivirus polymerase (Fields et al., 2001). Recombination can occur in BVDV when virions having different genomic sequences coinfect the same host cell, allowing genetic crossover resulting in a hybrid BVDV strain (Moenning et al., 1993; Becher et al., 1999a). Sequence analyses of cytopathic strains of BVDV, as discussed in more detail below, suggest genomic hot-spots for viral recombination at the border of NS2 and NS3, though recent cloning experiments using replicons expressing NS3 argue for functional selection of mutations or recombination events, based on a highly conserved region within the NS3 that is critical for effective viral replication (Tautz and Thiel, 2003). Genetic recombinations be-

tween noncytopathic and cytopathic (Ridpath and Bolin, 1995b; Fritzemeier et al., 1997; Nagai et al., 2003), BVDV type 1 and type 2 (Ridpath and Bolin, 1995b), BVDV persistent infection strains and vaccine strain (Ridpath and Bolin, 1995b; Becher et al., 2001), and between BVDV and host RNA (Becher et al., 2002; Baroth et al., 2000; Mendez et al., 1998; Meyers et al., 1989; Meyers et al., 1991a; Meyers et al., 1998; Qi et al., 1998; Ridpath and Bolin, 1995a; Ridpath et al., 1994; Ridpath and Neill 2000; Rinck et al., 2001; Tautz et al., 1996; Tautz and Thiel., 2003) have been well documented. Cellular insertions include duplicated viral sequences, host ubiquitin or ubiquitin homologs, ribosomal ubiquitin gene fusion protein, host mRNA encoding a DnaJ or J-domain-regulatory proteins, and multiple microtubule-associated proteins. The insertion and recombination events can occur at different sites along the viral genome (Desport et al., 1998; Meyers et al., 1992; Meyers and Thiel, 1996); however, those involving the NS2/3 coding sequences have been most extensively studied, based on association with the generation of cytopathic BVDV (Meyers et al., 1996; Fields et al., 2001). The genetic modifications associated with NS2-NS3 cleavage tend to be associated with two specific sites located within 54 base pairs of each other and within BVDV NS2/NS3 (Ridpath and Neill, 2000).

CYTOPATHOLOGY

Cytopathic strains of BVDV evolve from noncytopathic BVDV by mutation, with specific genomic rearrangements varying considerably between different cytopathic strains of the virus (Becher et al., 1998a; Kummerer and Meyers, 2000; Meyers et al., 1991a, 1991b; Meyers et al., 1998; Muller et al., 2003; Nakamura et al., 1997; Qi et al., 1992; Qi et al., 1998; Ridpath and Bolin, 1995a; Ridpath and Neill, 2000; Tautz et al., 1993; Tautz et al., 1994; Tautz et al., 1999; Tautz et al., 2003). The genomic rearrangements generating cytopathic BVDV—including duplications of the BVDV genome, insertion of cellular sequences at the junction of NS2 and NS3, or in-frame deletions—primarily affect the coding region for the NS2-3 polypeptide and result in the production of NS3. Cleavage of NS2/NS3 occurs by different theoretical strategies, dependent on the strain of BVDV and may include introduction of sequences that form new cleavage sites, introduction of sequences with autocatalytic activity, introduction of conformational changes signaling cellular protease, or activation of latent protease activity directly encoded by NS2 (Fields et al., 2001; Meyers

and Thiel, 1996; Ridpath and Neill 2000; Rinck et al., 2001). NS3 production, and associated BVDV cytopathogenicity, have also been generated by point mutations in the region encoding NS2 (Kummerer et al., 1998; Kummerer and Meyers, 2000), upstream of the NS2/3 region (Kummerer et al., 1998), or through a specific mutation in the region encoding NS4b (Qu et al., 2001). The majority of cytopathic BVDV characterized in the literature appear to be associated with genomic insertions, of either host cell sequences or duplications of the viral genome. A relatively small number of the characterized cytopathic BVDV sequences appear to arise through recombination between BVD viruses having different genomic sequences. Proteolytic processing associated with generation of BVDV cytopathology is further discussed in Chapter 11. In addition to BVDV superinfection with a cytopathic and noncytopathic BVDV pair, spontaneous cases of mucosal disease arise when noncytopathic persistent BVDV mutates or undergoes recombination, as reviewed in Chapter 8 on reproductive disease and persistent infections.

DEFECTIVE INTERFERING PARTICLES AND CYTOPATHOLOGY

Viral genomes having deletions or truncations, which can replicate with the support of a helper virus or helper virus-coreplication, are referred to as defective interfering (DI) particles (Huang and Baltimore, 1970). BVDV DI particle have been associated with cytopathology in vitro and mucosal disease in vivo. A BVDV DI particle, approximately 4.3 kb smaller than the corresponding noncytopathic BVDV virus and lacking all structural genes plus the amino-terminal region of the nonstructural NS3 protein, was initially described from a persistently infected calf in 1994 (Tautz et al., 1994). Other BVDV DI, with slightly different deletions but lacking all structural genes, have been described (Kupfermann et al., 1996), and at least one (DI9c) has been shown to function as an autonomous replicon (Behrens et al., 1998).

DI particles have been implicated in the onset of mucosal disease in cattle persistently infected with a noncytopathic BVDV, potentially through recombination events involving structural proteins of the helper noncytopathic virus (Kupferman et al., 1996). Theoretically, the structural genes of the persistent virus would not be recognized by the immune surveillance of the host, allowing the recombinant BVDV virus to replicate while escaping the host im-

mune response. The existence of DI in naturally occurring disease has been confirmed from animals with mucosal disease; however, DI are difficult to detect due to low virus yields, and they also can get lost easily during virus isolation and plaque purification (Meyers and Thiel, 1996). DI particles are typically observed in vitro only during cell culture passages with a high multiplicity of infection (Meyers and Thiel, 1996).

PROTEOLYTIC PROCESSING

The single BVDV polyprotein translated from the open reading frame (ORF) undergoes proteolytic processing to derive at least 11 viral proteins (Meyers and Thiel, 1996). BVDV structural proteins are located within the N-terminus third of the polyprotein, followed by a single non-virion protein (p7), and nonstructural proteins that comprise the remainder of the polyprotein. Proteolytic processing of the polyprotein is mediated by both host and viral enzymes, including a viral autoprotease (N^{pro}) unique to pestiviruses, host signal peptidase, and viral serine protease (Fields et al., 2001). BVDV proteolytic processing and function of the structural and nonstructural proteins are discussed in detail in Chapters 3 and 11.

REGULATION OF TRANSLATION AND REPLICATION

Tight regulation of translation and replication is required during the BVDV lifecycle, since the same positive-sense RNA is used as template for both processes. Structural and functional studies of the 5' terminal fragment of the BVDV genome, including the IRES site, demonstrate secondary structure that could enable viral RNA to switch from a translation to a replication cycle (Yu et al., 2000). Accumulation of viral proteins, such as NS5A and NS5B, which can inhibit IRES-dependent translation may also provide a regulatory mechanism for the translation-to-replication switch. Kinetic analysis of translation and replication using BVDV full-length and replicon genomes indicate that regulation of the translation-to-replication switch is not specific for the BVDV IRES, but may result from regulatory proteins interacting with viral genome outside the IRES or from interaction between viral and host translation protein complexes (Li and McNally, 2001). Alternately, competition for replicase and ribosome-loading on the same template molecule may serve to regulate the switch.

The asymmetric replication of the BVDV genome is consistent with other positive-sense RNA viruses,

where an excess of newly synthesized positive-sense RNA compared to negative-sense RNA is generated. A close functional control appears to exist between generation of the polyprotein and generation of replication complexes. The replicase complexes, formed by association of new viral nonstructural proteins plus cellular components with the 3' end of the genomic RNA, act to catalyze synthesis of a low copy number of negative-strand RNA templates for the transcription of an excess of positive-strand RNA progeny (Behrens et al., 1998; Yu et al., 2000).

VIRION ASSEMBLY AND EXOCYTOSIS

Little information is available on the assembly and release of virions from infected host cells. BVDV virions appear to mature in intracellular vesicles at the Golgi apparatus or endoplasmic reticulum where the lipid envelope is acquired through budding into the vesicle lumen. Virus maturation, including conformational stabilization through glycoprotein folding of E1-E2 (Branza-Nichita et al., 2001) and associated transport to the cell surfaces is mediated by host cell enzymes and processes (Zitzmann et al., 1999, Branza-Nichita et al., 2001, Durantel et al., 2001). The intact virions are released by budding into the cisternae of the endoplasmic reticulum, followed by exocytosis (Grummer et al., 2001; Bielefeldt-Ohmann and Block, 1982; Gray and Nettleton, 1987) with detection reported as early as 8 hours postinfection (Nuttall, 1980).

QUASISPECIES AND POPULATION GENETICS

The RNA-dependent RNA polymerases that catalyze RNA replication and reverse transcription have minimal proofreading activities, resulting in error rates tens of thousands times greater than those encountered during DNA replication (Domingo and Holland, 1997; Holland et al., 1992; Malet et al., 2003; Moya et al., 2000). Because mutation is a frequent event, an RNA virus population does not represent a homogeneous clone, but is a "cloud of mutants" clustered around the most frequent viral sequence (Moya et al., 2000). The related, but nonhomogeneous populations have been identified as quasispecies (Eigen, 1993). Although the theories of quasispecies and population genetics differ in mathematical modeling and the contributions of point mutations to viral evolution (Page and Nowak, 2002; Moya et al., 2000; Domingo, 2003), it is agreed that the target of natural selection is not a single fittest genotype, but a distribution of genotypes around a master sequence. The existence

of quasispecies among BVDV strains reflects the high replication rate of the virus, as well as the lack of proofreading capacity of the viral RNA-dependent RNA polymerase. BVDV is able to effectively evade emerging humoral and cellular immune responses (Bolin et al., 1991), which may be a function of BVDV quasispecies generation in infected hosts. Among BVDV, point mutations occurring approximately once per 10 kb would be equivalent to one or more mutations per BVDV viral replication cycle and logically explain the existence of multiple distinct, but closely related, genetic variants of BVDV. BVDV quasispecies have been recovered (Becher et al., 1999a; Jones et al., 2002) from persistently infected cattle, though other studies have reported apparent stabilization of the genome in persistent infection (Edwards et al., 1991; Edwards and Patton, 1995; Hamers et al., 1998, Hamers et al., 2001). Stabilization of the antigenic, if not genetic, character of the virus is consistent with the theory of immunologic elimination of BVDV variants in PI animals. Stabilization, or lack of significant genetic change in the genome of BVDV isolated from PI cattle over time, is also consistent with observations of herd-specific strains of BVDV (Paton et al., 1995).

REPLICATION SITES

Similar to most RNA viruses, BVDV replicates in the cellular cytoplasm. In studies aimed at subcellular localization of BVDV replication, nonstructural proteins NS2-3 and NS3 were found in association with the cytoplasmic face of the endoplasmic reticulum, but not with the Golgi apparatus or lysosomal membrane, suggesting that replication occurs on the cytoplasmic side of the endoplasmic reticulum (Zhang et al., 2003). Both cell and species tropisms are seen with BVDV. Though BVDV can infect a wide variety of cell types, both in vitro and in vivo, there is an apparent predilection for cells of the immune system, including T cells, B cells, monocytes, macrophages, and dendritic cells (Sopp et al., 1994). The biotypes of BVDV behave differently, with noncytopathogenic strains having tropism for leucocytes, lymphoid tissues, parotid gland, proximal colon, and respiratory tract; cytopathogenic strains are generally associated with the gastrointestinal tract (Greiser-Wilke et al. 1993; Liebler et al., 1991) and replicate in the ovaries (Grooms et al., 1998).

SUMMARY AND CONCLUSIONS

Important genetic diversity occurs among BVDV isolates, as evidenced by point mutations, deletions, genetic recombination among virus strains, and by

integration of segments of the host genome. Each BVDV infection represents an encounter between the genetic makeup of the virus and that of its host, allowing BVDV to respond and take advantage of its ability to generate and exist as quasispecies. Knowledge of the replication cycle allows not only understanding of the epidemiology and a wide range of diseases caused by BVDV infection, but also provides opportunities for development of effective detection, vaccination, and control strategies based on mediation of receptor recognition, interference with virus-specific replication, and blockage of viral protein synthesis and exocytosis.

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5

Virus Transmission

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INTRODUCTION

Transmission of bovine viral diarrhea virus (BVDV) in cattle follows the infectious disease epidemiology characteristics of many viral infections, with a notable exception: Infection with BVDV can manifest in two patterns for the infectious period of the disease, depending on when the animal became infected. Infected animals can shed the virus transiently, following an acute infection acquired postnatally, or they can shed the virus persistently, following congenital infection acquired before 120–150 days of gestation. This chapter reviews concepts of disease transmission in the context of BVDV followed by specific discussion of routes of transmission and factors that may affect virus transmission from animals with an acute or a persistent infection. The chapter concludes with a brief summary of general considerations for minimizing transmission of the virus in cattle populations.

INFECTIOUS DISEASE EPIDEMIOLOGY IN THE CONTEXT OF BVDV TRANSMISSION

FACTORS AFFECTING BVDV TRANSMISSION

Conceptually, four main factors can be expected to influence the transmission of infectious agents such as BVDV (Haloran, 1998). The first factor is the infectiousness of the virus strain, given the exposure dosage and route of infection, referred to as the coefficient of infectiousness (β). The latter is a measure of the probability that infection will be transmitted to a susceptible animal following contact with an infectious animal. As discussed later, animals persistently infected with BVDV generally shed more virus over a much longer period of time than animals with an acute infection, so the coefficient of in-

fectiousness would be higher for PI animals than for animals with an acute infection. The second factor is the number of adequate contacts per time period (k) between infectious and susceptible animals. An adequate contact is one that would be sufficient for transmission to take place, such as direct nose-to-nose contact between an infectious animal and a seronegative, susceptible animal. The third factor is the duration of the infectious period (d) for the specific host, or, alternatively, the prevalence of infectious animals in a herd during a specified period. The fourth factor is the presence of truly susceptible animals that lack specific or cross-reacting serum neutralizing (SN) antibodies (humoral immunity) and/or cell-mediated immunity necessary to prevent infection.

These factors can be used to estimate rates of virus transmission in a susceptible population. One approach to estimating the rate or force of transmission is by the use of basic reproduction number (R_0), referred to as “R-not,” which represents the expected number of new infections resulting from contact with an infectious index case animal, and is calculated as $R_0 = \beta dk$. For example, suppose, hypothetically, that the probability of a susceptible weaned calf becoming infected per adequate contact with an acutely infected animal in a feedlot is 0.02. That is, if an adequate contact was made between an acutely infected animal and 100 susceptible animals, the virus would be transmitted successfully to 2 animals. Suppose also that the number of contacts made, on average, is 50 per day and the duration of infectiousness is 4 days, then $R_0 = \beta dk = (0.02/\text{contact})(4\text{-day duration})(50 \text{ contacts/day}) = 4$. A value of 4 would indicate that the acutely infected calf (index case) would infect 4 susceptible calves, and each of them in turn would infect 4 other calves.

As transmission proceeds through a herd, how-

ever, infected animals become immune and the number of susceptible animals decline. As the number of susceptible animals decline, the likelihood that an infectious animal will come in contact with a susceptible animal will decrease, and the R -value will decline with each newly infected animal. Eventually, the number of susceptible animals becomes so small that the transmission rate will decline to $R = 1$, referred to as the *endemic state*, in which each new infection creates only one more new infection. As the number of susceptible animals declines further, R will become less than 1 and new infections will stop because transmission is no longer supported by a sufficient number of susceptible animals. Transmission will not continue unless additional susceptible animals are added to the herd, the immunity wanes, the contact rate is increased, the duration of infectiousness increases (i.e., a recrudescence or extension of shedding), or pathogenicity of the virus changes (i.e., mutation).

Because β is often unknown, an alternative approximation for R_0 is $R_0 = 1 + L/A$, where L is the average or median life span of animals in a herd and A is the average or median age of animals at the time of infection. Suppose, for example, the median lifetime of a cow on dry-lot dairies is 40 months and half (median) of the animals are infected with BVDV by 8 months of age (Rush et al., 2001), then $R_0 = 1 + 40/8 = 6$. Thus, one would expect that in a fully susceptible population, introduction of 1 infected animal would result in transmission of infection to 6 susceptible animals, which in turn would transmit infection to other susceptible animals, and so on. This somewhat hypothetical example does not distinguish between persistent and acute infections.

Alternatively, β can be estimated using the formula for incidence of BVDV infection, I_I , along with available data and reasonable assumptions. Incidence provides a measure of transmission by estimating the rate at which new BVDV infections occur, or the proportion of susceptible animals that become infected during a specified time period. The formula for I_I is $I_I = \beta Pk$, where β is the unknown probability of infection given a contact (coefficient of infectiousness), P is the assumed prevalence of infectious animals for a given time period, and k is the contact rate for the given time period (Haloran, 1998). Suppose incidence is 0.5% per day for weaned calves in a feedlot environment (Taylor et al., 1995; Rush et al., 2001), k is about 25 contacts per day, and the prevalence of PI animals is 1% (assuming that all PI animals are infectious and ignoring the prevalence of acutely infectious animals),

then $\beta = (0.005)/(25)(0.01) = 0.02$. After substituting this value for β into the formula for R_0 , and assuming an infectious period of at least 100 days for animals with PI, $R_0 = (0.02)(25)(100) = 50$. Because these formulas assume random mixing, constant duration of infectiousness, and a constant coefficient of infectiousness, they should be used to obtain a general sense for the rate of transmission. The main benefit of these equations is that they provide a conceptual context for key elements in the transmission of BVDV.

MEASURING THE EFFECT OF VACCINATION ON TRANSMISSION

In addition to characterizing key forces influencing BVDV transmission (i.e., probability of infection, contact rate, and duration of infectiousness), estimates of R_0 and I_I can be used as a benchmark for assessing vaccine efficacy required to control infection. Suppose, for example, that $R_0 = 4$, whereby introduction of an acutely infected animal into a completely susceptible herd or group of animals will result in 4 new infections. If the proportion of the animals, p , represents those animals that have been effectively immunized and that are not susceptible is 0.5, 1 new BVDV infection will result in only 2 new infections rather than 4, because half of the contacts will be immune. In planning a vaccination program, we can use R_0 to estimate p , the proportion of the herd that must be immunized to prevent transmission within the herd. If $p \times R_0$ animals will not become infected because of vaccination, the average number of animals that will become infected by the primary case is $R_0 - (p \times R_0)$.

In order to prevent transmission of infection, the number of secondary cases resulting from the primary case will need to be less than 1, or $R_0 - (p \times R_0) < 1$. This is equivalent to $R_0 - 1 < (p \times R_0)$ or $p > 1 - 1/R_0$, which is the formula for vaccination protection (Giesecke, 2002). In other words, if $R_0 = 4$, then the proportion of the herd that will need to be effectively immunized by vaccination (assuming the vaccine is 100% effective in immunizing an animal against the particular virus strain) to prevent transmission from acutely infected animals is $1 - 1/R_0 = 1 - 1/4$, or 75%. If the R_0 for the strain causing the acute infections was 2, rather than 4, only 50% of the herd would need to be immunized.

TRANSMISSION UNDER THE INFLUENCE OF HERD IMMUNITY

Herd immunity implies that the overall immunity of the herd is greater than the sum of immunity enjoyed

by individual animals in the herd. In other words, a nonimmune, completely susceptible animal enjoys some immunity from infection if it is in a herd with animals that possess immunity, where immunity may have been acquired by natural infection with a field strain, through vaccination, or from colostral antibodies. Several factors can contribute to herd immunity. Transmission within a herd or group of animals with a high proportion of immune animals is slower, as shown above using R_0 or I_1 , than for herds with a low proportion of immune animals. Thus, a susceptible animal in a herd with a high proportion of immune animals will have a lower risk of infection than if it were in a herd with a low proportion of immune animals. Individual animal immunity also can affect the dynamics of transition from one disease state (i.e., latent, infectious, noninfectious) to the other and the infectious dose needed to produce infection. For example, animals with little or no immunity can be expected to have a shorter latent period and to require a lower dose of virus to become infected, compared with animals with immunity.

Although specific data are not available for BVDV herd immunity, some generalizations are possible by applying what is known about the infectiousness of the disease. For example, depending on strain variation, cell-mediated or humoral immunity acquired from exposure to one strain of BVDV may reduce the amount of virus shed and/or duration of shedding, and thus reduce β and d , if the animal is infected with a different strain of the virus (Howard et al., 1989; Brock et al., 1998; Grooms et al., 2001). Generally, immunity to most infectious agents is not likely to be absolute; infection may be acquired by animals with some marginal or incomplete spectrum of immunity if exposed to a sufficiently large dose of the agent. This is particularly true in the case of BVDV because of the presence of high antigenic diversity among BVDV strains (Dubovi, 1992; Ridpath, 2003). Low levels of immunity might protect against low doses of a reasonably homologous strain, but higher levels of immunity would be required to protect against high doses of the virus, particularly if it is a heterologous strain. If an animal with partial immunity becomes infected, however, it may not necessarily shed as much virus and not for as long, when compared to a nonimmune animal (Howard et al., 1989; Brock et al., 1998; Grooms et al., 2001). Generally, acute BVDV infection of animals with some immunity can be expected to manifest in shorter periods of shedding and lower doses of the agent being shed, which subsequently would reduce the probability of transmission to a fully sus-

ceptible animal. The extent to which viral shedding is reduced in PI animals with SN antibodies is not known. However, the transient cessation of viremia in some PI animals after consuming BVDV colostral antibodies or with active SN antibodies could suggest that viral shedding might also be reduced, at least temporarily.

When herd immunity is present, virus transmission is less likely. This is because herd immunity results in a reduction in the number of animals that are susceptible to viral infection. Thus, the probability that the agent will be transmitted to a susceptible animal during an “adequate” contact is reduced, as indicated by a diminished value for β in the equations above for R_0 and incidence. In addition, the reduced duration of shedding (d), or reduced prevalence of infectious animals (P), will further diminish the force of transmission through a herd, as indicated by the equations for R_0 , and the diminished incidence would not only reduce disease caused by BVDV but would also reduce virus transmission by limiting the amount of virus shed from infected animals.

HORIZONTAL AND VERTICAL TRANSMISSION OF BVDV

Transmission of BVDV can occur vertically, resulting in congenital infection of the fetus, or horizontally after birth, sometimes referred to as *postnatal transmission*. Transmission of BVDV to the fetus before 120–150 days in gestation may result in abortion, resorption, or stillbirth. Surviving fetuses will be born as BVDV-infected calves. The BVDV infection will persist for the life of the animal and will result, presumably, in the continuous shedding of the virus into the environment. Thus, PI animals are believed to serve as a reservoir of BVDV. In utero transmission after about 120–150 days of gestation, when fetus becomes immune-competent, will result in a congenital infection characterized by abortion, stillbirth, congenital defects, or the birth of a live, normal-appearing calf. Horizontal (postnatal) transmission results in an acute infection sometimes referred to as *transient infection (TI)*, varying in severity from inapparent or mild signs to life-threatening disease. Although animals that have acquired an acute BVDV infection may remain infected for some time, as indicated by prolonged duration of SN antibodies for a year or more, it is generally believed that they shed the virus only transiently for a week or less. Unfortunately, the requisite long-term data are not available to confirm or deny subsequent recrudescence of shedding. Several reviews of BVDV have addressed specific aspects of BVDV infection

(Duffell and Harkness, 1985; Radostits and Littlejohns, 1988; Harkness, 1987; Brownlie, 1990; Houe, 1995; Radostits et al., 1999).

CONGENITAL TRANSMISSION RESULTING IN PI

There are two ways in which a fetus can acquire a persistent infection. One is by transmission of the virus from a PI cow to her fetus, which very likely will result in PI calf. The other way is by acute infection of the dam in the first 120–150 days of gestation during which it can transmit infection to the fetus. The rate at which PI animals are created in a herd depends on the prevalence of PI among pregnant cows and on the proportion of cows acquiring an acute BVDV infection during the first 120–150 days of gestation. The ability of BVDV to infect embryos prior to attachment may be limited, as suggested by studies showing only one of six fetuses exposed before 60 days developed into PI calves (Moerman et al., 1993). In contrast the generation rate of PI animals was significantly higher when fetuses were exposed after 45–60 days gestation (Liess et al., 1984).

CONGENITAL TRANSMISSION AFTER 120–150 DAYS IN GESTATION

Little information exists on the rates of congenital transmission in fetuses after they become immune-competent. Two large studies of midwestern slaughterhouses found that 20% of the fetuses from culled cows had evidence of BVDV infection (Bolin et al., 1991b; Bolin and Ridpath, 1998), some of which appeared to be attributable to cytopathic vaccine strains. As shown in a recent field study of large dairies, congenital transmission after 120–150 days gestation may occur in 10% or more of vaccinated dairy cows (Muñoz-Zanzi et al., 2003a). This estimate represents only those calves that are born alive and probably would be considerably higher if congenital infections in aborted fetuses and stillborn calves are also included. Congenital transmission of BVDV after 120–150 days in gestation, which is indicated by the presence of precolostral BVDV-neutralizing antibodies in calves at birth, has recently taken on new importance. Calves congenitally infected with BVDV were more likely to develop severe diseases in the first 4–5 months of life (Muñoz-Zanzi et al., 2003a) and to experience breeding difficulties as heifers (Muñoz-Zanzi et al., 2003b).

HORIZONTAL TRANSMISSION AND ACUTE INFECTION

Acute infection results from horizontal transmission of the virus to a susceptible animal following ade-

quate contact either with an acutely infected animal that is in the infectious (shedding) phase of the disease or with a PI animal that continuously sheds the virus. Acute infection can be acquired through various routes of transmission, including direct nose-to-nose contact, fomites contaminated with the virus, and vaccination with a modified live virus (MLV) (Duffell and Harkness, 1985; Harkness, 1987; Brownlie, 1990; Houe, 1995; Radostits and Littlejohns, 1988; Radostits et al., 1999).

ROUTES AND MEANS OF BVDV TRANSMISSION

Generally, one can expect BVDV to be shed in most excretions and secretions, including tears, milk, saliva, urine, feces, nasal discharge, and semen of acutely infected animals during the transient, infectious phase of the disease, as well as those of most if not all PI animals (Houe, 1995). Some key routes of transmission pertaining to current management conditions are discussed in the following sections.

TRANSMISSION VIA SEMEN

Semen from PI and acutely infected bulls contains large amounts of BVDV, although semen from acutely infected bulls typically would have much less virus than semen from PI bulls (Brock et al., 1991; Voges et al., 1998). Transmission of BVDV via semen can result in an acute infection or in the development of PI, or both. The potential for establishment of persistent infection in calves sired by a PI bull was illustrated in a study in which all 12 seronegative heifers developed acute BVDV infection following insemination with semen from a PI bull (Meyling and Mikel Jensen, 1988). One of the 12 calves (8%) developed persistent infection, indicating a significant potential not only for acute transmission of BVDV from PI bulls but also for the establishment of PI animals. Even though only 1 of 12 calves developed PI, a transmission rate of 8% would suggest that natural breeding by PI bulls could contribute significantly to an accumulation of PI animals in large herds.

The risk of calves developing persistent infection may be high in herds using a large number of bulls in natural breeding, particularly in large dairy herds where each bull may service several hundred cows per year and in herds with a significant proportion of susceptible, antibody-negative females. In one case, a single PI bull was found to have transmitted BVDV to 55 cows, resulting in at least 2 PI animals, and was responsible for spreading BVDV to several clean herds (Kirkland et al., 1994). In contrast, in-

semination of 73 heifers with semen of a bull with acute BVDV infection resulted in virus transmission to only 3 of the animals, but a second cycle of transmission appearing 29 days after insemination resulted in 8 of 15 of the heifers that were subsequently followed becoming infected.

Bulls that acquire an acute BVDV infection also can shed the virus in the semen for several days after cessation of viremia, perhaps up to 14 days after infection, even though semen quality may remain normal (Kirkland et al., 1991; Kirkland et al., 1994). The much lower amount of virus found in the semen of acutely infected bulls, as compared with that in PI bulls, and the low rate of shedding of BVDV in acutely infected bulls (Whitmore et al., 1978), would suggest a low risk of BVDV transmission via semen from acutely infected bulls, compared with that from PI bulls. In one reported case, however, BVDV was shed persistently for at least 11 months in an apparently acutely infected young bull (Voges et al., 1998), suggesting that some acutely infected bulls may pose the same threat as PI bulls. Thus, use of semen from bulls with acute BVDV infection could pose a substantial risk of transmission to susceptible cows, with possible development of PI in their fetuses, as previously illustrated (Kirkland, et al., 1997).

The high prevalence of PI (0.8%) found among well-screened bulls at artificial insemination centers (Howard et al., 1990) and the high rate of PI in embryo transfer (ET) animals (>2%) (Hietala et al., 2000) suggest that bulls selected for breeding, particularly ET bulls, can be expected to have a higher probability of PI than the general population of cattle. Coupled with the high rate of contact with females, therefore, bulls can pose a significant risk of infection, either as a PI animal or temporarily as an acutely infected animal. Recommendations for reducing bull-related transmission include routine testing of bulls for PI, testing the semen for virus, and vaccination (Brock et al., 1991; Voges et al., 1998).

TRANSMISSION VIA EMBRYO TRANSFER

Embryo transfer can constitute an important means of BVDV transmission to valuable genetic stock. The virus can be transmitted to the fetus if the donor has acute or persistent infection and the embryo is not adequately washed, if the recipient has acute infection or PI, or if the fetal calf serum (FCS) used to wash the embryo contains BVDV. The replication and persistence of BVDV in *in vitro* embryonic production systems appears to be strain-specific

(Givens et al., 2000). Large amounts of virus have been found in serum (10^6 TCID₅₀/ml), vaginal mucous (10^6 TCID₅₀/ml), urine (10^4 TCID₅₀/ml), uterine flush medium (10^2 TCID₅₀/ml), and feces (10^4 TCID₅₀/ml) of a PI donor heifer (Brock et al., 1991). In addition, several studies have documented BVDV contamination of commercial FCS. In one study, 10% of irradiated lots and 62% of nonirradiated lots of FCS were contaminated. Heat inactivation of serum at 56°C for 30 min was not considered to be a reliable method to eliminate BVDV from sera (Rossi et al., 1980). The virus also was found in 20.6% of slaughterhouse fetuses, which constitute the main source of FCS (Bolin et al., 1991b). BVDV was found in 20.3% of 1000 lots of FCS harvested by a commercial supplier from an abattoir that killed beef and dairy cattle from three states (Bolin and Ridpath, 1998). Thus, even if donors and recipients are not infected with BVDV, use of untested FCS may result in transmission of BVDV to an embryo and establishment of PI. Embryo transfer also can result in back-transmission of BVDV from an infected or contaminated embryo to a susceptible recipient animal. It has been proposed that maternal families of animals with PI may be established if future breeder animals acquire PI as a result of BVDV transmission through embryo transfer (Brock et al., 1991).

TRANSRECTAL TRANSMISSION

Rectal transmission of BVDV was demonstrated following the palpation of eight seronegative heifers, all of which seroconverted after a single rectal palpation of the uterus and ovaries, using the same glove that had been used to palpate an animal with PI. Virus was isolated from five of the heifers, and all eight showed mild signs of clinical BVD. The results suggest that transmission to seronegative animals following rectal palpation with the same glove as used for a PI animal could be an important means of transmitting BVDV (Lang-Ree et al., 1994), and of establishing PI if cows are palpated before 120 days in gestation.

IATROGENIC, FOMITE, ENVIRONMENTAL, AND INSECT TRANSMISSION

The BVD virus can survive in a cool, protected environment for several days or even weeks (Houe, 1995). Consequently, fomite or iatrogenic transmission can occur if susceptible animals are exposed to feed or equipment (e.g., nose tongs, halters, milk bottle nipples, balling guns, etc.) previously used on animals with PI or acute infection (Radostits and

Littlejohns, 1988; Gunn, 1993; Houe 1995; Radostits et al., 1999). Transmission to seronegative animals also is possible by reusing the same 19 gauge needle within a few minutes after it has been used on a PI animal, but viable virus may remain in the needle for at least 3 days (Gunn, 1993). Environmental contamination of shared calving pens by uterine discharges from PI or acutely infected cows may create a common source of exposure for other cows and for newborn calves that lack colostral protection (Radostits and Littlejohns, 1988).

Environmental conditions that favor crowding and aerosol transmission would increase the likelihood of BVDV transmission from acutely infected, coughing calves with a respiratory form of BVDV infection, particularly that caused by BVDV type 1b (Baule et al., 2001). It also is possible for flies to mechanically transmit the virus to susceptible animals. The BVD virus was isolated from face flies (*Musca autumnalis*) feeding on the nose and face of a PI animal (Gunn, 1993). In another study, stable flies (*Stomoxys calcitrans*), horseflies (*Haematopota pluvialis*), and head flies (*Hydrotaea irritans*) force-fed for 10–15 minutes on a PI animal, were able to transmit infection to susceptible animals (Tarry et al., 1991).

TRANSMISSION VIA MILK AND COLOSTRUM

Milk from PI cows can be expected to contain BVDV and thus will serve as a source of virus for calves fed unpasteurized waste milk from sick or treated cows, as is practiced commonly on large calf-raising operations. Colostrum from cows with persistent or acute infection may or may not contain infectious BVDV, depending on whether cross-reacting neutralizing antibodies are present.

TRANSMISSION VIA MODIFIED LIVE VIRUS VACCINE

Iatrogenic transmission of BVDV to the fetus, resulting in CNS lesions, teratogenic defects, and death, can occur if susceptible females are vaccinated with a MLV BVDV vaccine during pregnancy (Orban et al., 1983; Liess et al., 1984). A survey of fetuses harvested from midwestern slaughterhouses found cytopathic strains of BVDV identical to those used in some MLV vaccines (Bolin et al., 1991b), suggesting that fetal infection following BVDV MLV vaccination may not be uncommon. Vaccination with MLV BVDV also can be a highly efficient means of transmitting BVDV to the fetus and producing animals with persistent infection. An experimental study of congenital infection following MLV

vaccination found evidence of PI in all live-born calves of 9 seronegative cows vaccinated before 120 days in gestation, while fetuses of 12 other vaccinated seronegative cows were aborted, had congenital defects, or were stillborn (Liess et al., 1984).

Shedding of virus after vaccination can be minimal for some strains. In one study, a transient viremia lasted 3–10 days after vaccination, but no shedding was detected in nasal secretions or by exposing susceptible contact animals (Kleiboeker et al., 2003). In contrast, PI calves infected with BVDV type 1b and given a BVDV type 1a MLV vaccine shed the 1a vaccine strain of virus in nasal secretions for up to 28 days after vaccination (Fulton et al., 2003). These findings suggest that transient shedding of vaccine strains is possible and may represent a means of secondary transmission to pregnant animals in contact with vaccinated animals.

TRANSMISSION OF BVDV FROM INFECTED ANIMALS

Contagiousness, or the degree to which BVDV-infected animals can transmit infection following contact with a susceptible animal, can depend on multiple factors, which may include type of infection (PI or acute), the stage of an acute infection, presence of neutralizing antibodies, and the strain of virus and its associated virulence.

TRANSMISSION FROM PI ANIMALS

PI animals can be assumed to shed large amounts of BVDV in secretions and excretions most, if not all, of the time. Pregnant PI cows invariably transmit the virus to the fetus in the first 120–150 days of gestation, resulting in persistent infection of the fetus. Bulls with persistent infection will shed large amounts of the virus in their semen, while maintaining acceptable semen quality. An understanding that PI is transmitted from generation to generation along maternal lines (Radostits and Littlejohn, 1988; Brock et al., 1991) can be used in identification of PI animals by testing the dam, daughters, and sons of PI females.

In contrast to acute infection, PI animals are believed to continuously shed large amounts of virus, thereby posing a constant threat of exposure to susceptible animals. However, considerable data have accumulated to indicate that viremia in PI animals with SN antibodies that cross-react with the PI strain will cease for some period of time until antibodies wane (Howard et al., 1989; Palfi et al., 1993; Brock et al., 1998; Grooms et al., 2001). Although data on virus shedding are not readily available, the cessation

of BVDV viremia suggests that the amount of virus shed in secretions and excretions and the duration of shedding might also be diminished for some period. The existence of some PI animals that could remain undetectable through intermittent or low shedding would be a logical explanation for the unexpectedly prolonged and continued transmission in herds thought not to have any PI animals, and for which transmission was thought to be solely from acutely infected animals (Barber et al., 1985; Moerman et al., 1993). In light of these findings and the intermittent viremia in PI animals (Brock et al., 1998), it would be prudent for programs aimed at detecting PI animals and at reducing BVDV transmission to consider the possibility that PI animals may shed the virus intermittently, rather than continuously.

TRANSMISSION FROM ACUTELY INFECTED ANIMALS

The amount of virus shed and the duration of shedding by acutely infected animals very likely depend on the virulence of particular strains of BVDV and their propensity to replicate (Bolin and Ridpath, 1992), as well as on the presence and repertoire of SN antibodies (Bolin et al., 1991a). Animals infected acutely are believed to shed the virus briefly, perhaps for only a few days or weeks, depending on the strain of the virus (Duffell and Harkness, 1985; Niskanen et al., 2000). Under field conditions, where the vast majority of acute infections by indigenous strains in a herd are mild and subclinical, the duration of shedding may be only 1–2 days, or less. Failure to detect viremia by alternate-day PCR testing in unaffected dairy calves during a 4-week period preceding seroconversion suggests that viremia and shedding in acute infections can be very short-lived, perhaps less than 24 hours in calves experiencing no untoward signs of infection (Thurmond, unpublished observations).

In contrast to endemic BVDV infection and transmission of indigenous strains among cattle in a herd, the severity of disease and duration of shedding have been observed to be much different following the addition of new cattle to a herd. Clinical disease has been more severe and the virus has been shed for longer periods both in newly purchased, out-of-state cattle following week-long exposure to their new herdmates and in some of the herdmates in contact with the new replacements (Thurmond, unpublished observations). A possible explanation is that the animals became infected with new strains that were sufficiently different from those that directed their repertoire of cell-mediated and SN antibody immu-

nity such that the infecting viruses were able to produce severe disease, with viral shedding extending for several days or weeks. Similar observations were made in an experimental study where acute infection with virulent strains producing severe clinical disease resulted in more extensive shedding of virus for well over a week, compared to infection with strains that caused only mild disease (Kelling et al., 2002). Thus, the potential for shedding and transmission from acutely infected animals should be expected to vary considerably, depending on cross-protection acquired from other exposures, including vaccines (Bolin et al., 1991a), and the virulence of the virus.

As noted elsewhere (Brownlie, 1990), little attention has been given to the presumed latency of acute BVDV infections. A prevailing assumption has been that acute infections result only in transient shedding of the virus, perhaps as long as 14–21 days (Duffell and Harkness, 1985), but more typically between 1–2 days or less than a week, with no recrudescence of virus later on. Unfortunately, there are no reports of the requisite long-term follow-up studies necessary to confirm or deny such an assumption. The fact that SN titers persist long after viremia wanes indicates the virus is still replicating and being presented to the lymphoid tissue. Although seemingly unlikely, given the effect of SN antibodies in suppressing viral shedding, exacerbation of an acute infection with renewed shedding, perhaps from sequestered, protected sites, could explain transmission cycles and unusually long time (2–3 years) for all animals in some small herds to become infected (Barber et al., 1985; Moerman et al., 1993).

It is not known with confidence whether, under certain conditions, the latency of acute infections that follows an initial transient shedding can be broken, allowing the virus to be shed again. For example, in one study, a young bull that had acquired an acute BVDV infection, perhaps at 10–11 months of age when the blood testicle barrier was being developed, was observed to persistently shed the virus in his semen for at least 11 months thereafter. Virus could not be detected in serum, buffy coat, or other organs except the testicles, but high SN antibody titers were maintained to the homologous virus (Voges et al., 1998). The fact that this case resembles an acute infection, rather than persistent infection, would suggest the possibility that acutely infected bulls could persistently shed virus in the semen and yet maintain high antibody titers with no detectable virus in the serum or buffy coat. The authors recommend that as part of routine screening of

bulls for BVDV, semen should be tested for the presence of the virus.

The recurrence of shedding in acutely infected animals also may depend on the biotype of the virus. Intermittent nasal shedding of the virus was observed in animals experimentally infected with a cytopathic strain (Fray et al., 1998). Others have detected cytopathic virus in tissues and nasal secretions, after clearance of the virus from the blood, while SN antibodies were still present (Baule et al., 2001). Authors of this study suggested that sequestration of cytopathic BVDV in acutely infected animals may present a source of virus for recurrent shedding.

EFFECT OF COLOSTRAL ANTIBODIES AND VACCINATION-INDUCED IMMUNITY ON TRANSMISSION

Passive or acquired immunity to BVDV can affect transmission in two ways. One is by potentially reducing the amount or duration of shedding in animals with an acute or PI infection, thus diminishing the amount and duration of infectiousness, as discussed above. Animals that shed lesser virus for a shorter period of time will enhance herd immunity by reducing the likelihood of susceptible animals acquiring the infection if they contact an infected animal. The other way immunity reduces transmission is by reducing the number of animals susceptible to infection where susceptibility exists on a continuous scale, rather than on an all-or-none scale. Animals with considerable immunity (i.e., high SN antibody titers to a diversity of strains) would be much less susceptible to a given infectious dose than animals with lesser immunity (i.e., low SN titers and limited antibody cross-reactivity). Immunity of varying degrees and duration can be acquired from colostrum antibodies, vaccination, and infection with field strains.

PREVENTION OF TRANSMISSION BY COLOSTRAL ANTIBODIES

Colostrum antibodies can offer protection from BVDV infection for the first 2–4 months of life, depending on the quality and quantity of colostrum consumed, after which antibodies decay to levels that may no longer protect against infection. A large study of calves on two dairies practicing good colostrum management estimated that SN colostrum antibody titers of half of the calves would decay to <1:16 by about 110 days of age for type 1 and 80 days of age for type 2 BVDV (Muñoz-Zanzi et al.,

2002). The rate of antibody decay determines the rate at which calves become susceptible to infection, and also to MLV BVDV vaccination, where SN antibody titers of 1:16–1:32 have been considered to offer protection against clinical signs associated with BVDV infection (Bolin and Ridpath, 1992; Howard et al., 1989).

Colostrum protection depends on the quality of the colostrum management program, including ingestion of first milking colostrum with a high specific gravity from multiparous cows immediately after birth. Cows contributing colostrum should be well immunized against BVDV, preferably with both type 1 and type 2 strains. Because BVDV colostrum antibodies can neutralize the BVDV in a MLV vaccine (Ellis et al., 2001), and thus diminish vaccine efficacy, it is necessary to know at what age colostrum BVDV antibodies can be expected to decay to <1:16. At this age, calves become more susceptible both to infection with BVDV and to an effective immunization with a MLV vaccine. Because colostrum antibodies of all calves do not decay at the same rate, the age of susceptibility that provides an early window for vaccination may vary by a month or more. Consequently, multiple vaccinations may be needed; an early vaccination to protect calves with little or no colostrum antibody and a later vaccination for calves in which antibodies decay more slowly. Coupled with timing of vaccination to avoid colostrum antibodies that may negate MLV vaccination is strategic vaccination before calves would begin to experience a risk of natural infection from herdmates.

The period of maximum transmission will vary from herd to herd, depending on the prevalence of PI calves and stocking rates. Transmission of BVDV in dairy calves in large, intensive dry-lot operations can begin at about 3 months of age and peak at about 7 months of age (Rush et al., 2001). Consequently, under conditions that foster such a high risk of infection, a narrow window for vaccination exists between the age when colostrum antibodies decay to <1:16 and when the risk of infection begins to accelerate.

PREVENTION OF TRANSMISSION BY VACCINATION

Most BVDV vaccination studies have targeted at how well vaccination might prevent or reduce disease caused by BVDV following experimental challenge with large doses of virus (van Oirschot et al., 1999) and not at how well vaccination might prevent infection. Because of the diversity of BVDV strains (Dubovi, 1992; Bolin et al., 1991a), the success of

vaccination aimed at increasing immunity and reducing the risk of infection and subsequent transmission will likely depend on the homology between the vaccine strain and the field strains present in the herd. The more homologous the strains, the more cross-protection will be achieved. One approach in using MLV vaccines is to preempt natural infections (Harkness, 1987), thereby controlling the timing of BVDV infection to strategically maximize immunity during critical periods of transmission. As discussed above, strategic vaccination of intensively managed calves attempts to induce an active immunity before transmission begins to increase at 3–4 months of age, but after colostral antibodies have decayed sufficiently so as not to neutralize the vaccine virus. Strategic vaccination also may be indicated before puberty and before heifers are bred to reduce conception failures associated with infection with field strains of BVDV (Muñoz-Zanzi et al., 2003b).

Another example of strategic vaccination is the common practice involving prebreeding vaccination with MLV vaccines or vaccination of pregnant cows early in gestation with killed vaccines. These strategies are intended to increase immunity, mainly by increasing the titer of SN antibodies, of the dams against acute infection and transmission of BVDV to the fetus before 120–150 days in gestation. The strategy of prebreeding vaccination with a MLV vaccine may reduce the risk of acquiring an acute infection in early pregnancy and subsequent development of PI in the fetus, depending on cross-protection between the strain of virus used in the vaccine and the wild strains circulating in the herd. However, complete protection against transplacental transmission to the fetus of cows given an MLV vaccine prebreeding may not be a realistic expectation. In one study, 2 of 12 (17%) antibody-negative heifers vaccinated 30 days before breeding with a MLV type 1 strain (NADL) gave birth to a PI calf after challenge exposure with a type 1 strain between 70 and 75 days in gestation (Cortese et al., 1998). In a similar study, PI was detected in 8 of 19 fetuses (42%) from cows vaccinated 45 days before breeding with a MLV type 1 strain (NADL) and challenged with a type 2 strain at 75 days of gestation (Brock and Cortese, 2001). The potential diversity and subsequent failure of cross-reactivity within genotypes also was observed whereby one or more of six calves vaccinated with either of two commercial vaccines, each containing a different BVDV type 1 strain, produced detectable antibodies against 10 cytopathic and 10 noncytopathic BVDV isolates,

but no calf produced antibodies to all 20 viruses (Bolin and Ridpath, 1989).

Further evidence for incomplete protection of the fetus by prebreeding vaccination was presented in a study in which transmission resulting in PI was not prevented in all cows vaccinated 3 weeks before breeding with a MLV type 1 vaccine; 7–15% of the fetuses of vaccinated cows challenged between 55 and 100 days in gestation with a selected heterologous type 1 strain developed PI (Dean et al, 2003). Use of a killed vaccine 2–3 weeks before breeding, also failed to prevent transmission of BVDV from cows to their fetus and establishment of PI (Bolin et al., 1991a). However, three reduced doses of killed vaccine prevented BVDV infection by respiratory challenge with the same vaccine strain (Howard et al., 1994), suggesting that killed vaccines may have a role in reducing transmission for some strains.

Further evidence for incomplete protection against congenital transmission was found in studies of newborn dairy calves from cows vaccinated with BVDV type 1 MLV vaccine. The study found that 10% of calves born alive had acquired a congenital BVDV infection, including infection with BVDV type 1 (Muñoz-Zanzi et al., 2003a), suggesting that considerable transplacental transmission can still take place in cows vaccinated once a year prior to breeding. These studies also illustrate the potential for genetic diversity of BVDV (Dubovi, 1992) for virus strains within a genotype and especially for strains with different genotypes (Ridpath, 2003). One should expect, therefore, that a vaccine containing only a type 1 strain of BVDV, for example, should not be expected to offer complete protection of the fetus against infection with other type 1 strains or necessarily any protection against infection with type 2 strains of the virus.

In a field study of natural horizontal transmission of wild strains of BVDV to dairy calves, vaccination with a MLV BVDV type 1 vaccine strain was estimated to prevent only 48% of infection with type 1 strains and effectiveness lasted only about 60 days. It is likely that presence of colostral antibodies was responsible for some of the incomplete protection. The vaccine, however, did not offer any detectable reduction in risk of infection with type 2 strains (Thurmond et al., 2001). In a similar study on another herd (Thurmond and Hietala, 2002), no difference in infection rates of BVDV type 1 was found between calves given a MLV vaccine containing both type 1 and type 2 strains and calves given a MLV vaccine containing only a type 1 strain. However, a significantly higher proportion of calves

given a vaccine with only one strain of BVDV type 1 became infected with BVDV type 2 than of those given the vaccine with both type 1 and type 2 strains. The efficacy in protecting against infection with BVDV type 2 was 64% for the vaccine with both type 1 and type 2 strains, compared with no protection offered by the vaccine with only a type 1 strain.

Field studies following the SN antibody titers of animals found considerable evidence for dual genotype natural infections, in which animals had evidence of infection with both type 1 and type 2 strains (Rush et al., 2001; Muñoz-Zanzi et al., 2002; Muñoz-Zanzi et al., 2003a), indicating that natural infection with one genotype does not necessarily protect against natural infection with the other genotype. Currently, most BVDV type 1 vaccines used in North America contain only type 1a strains, with the exception of one killed vaccine (Fulton et al., 2002). No vaccine trials have been reported that indicate the extent to which vaccines containing BVDV type 1a strains will protect against infection with type 1b strains, which have been found to be a predominant subtype in calves with respiratory disease (Fulton et al., 2002). These findings indicate that, under typical field conditions, the use of MLV vaccine with only a type 1 strain should not be expected to offer cross-protection against infection with type 2 strains, and suggest that vaccines employed to reduce transmission of both type 1 and type 2 strains of BVDV should contain both type 1 and type 2 strains.

INTRA-HERD TRANSMISSION

The rate at which BVDV is spread within a herd depends on the prevalence of PI animals, the rate of animal-to-animal contacts, the virulence of the virus strain(s), and the susceptibility of the cattle to new and indigenous strains in the herd. In studies of small, susceptible (unvaccinated) dairy herds with low density management involving seasonal grazing, transmission was reported to be slow, requiring 2–3 years for all adults to become infected (Barber et al., 1985), presumably in the absence of PI animal in the adult herd. In another similar herd, transmission was cyclical, with temporary increases in infection rates when acutely infected animals were mixed with susceptible animals, but also showing variation in transmission rates for cattle exposed to animals with PI (Moerman et al., 1993). Both studies concluded that transmission could be sustained for 2 or more years by shedding from acutely infected animals only, in the absence of PI animals, but that

transmission also can proceed unexpectedly slowly in a herd with PI animals. The R_0 was estimated in the latter study to be only 3.9.

In contrast, another study of transmission among animals in an extensive management system, with a stocking density of 67 animals/km², estimated R_0 to be 2.3 if no PI animals were detected and $R_0 = 35$ if the prevalence of PI in the herd was 1.2% (Cherry et al., 1998), suggesting a greater than tenfold increase in the transmission and risk of infection attributable to the presence of PI animals. To achieve eradication ($R = 1$), the authors concluded that if $R_0 = 32$, PI animals would have to be removed from the herd before 11 days of age. In herds without PI, a 2–3-year cycle was projected for fetal loss attributable to variation in the rate of acute infections. For cattle managed under extensive grazing conditions, where stocking density ranged from 0.2–1 animal/acre, no transmission was observed from animals with acute infection, but the incidence of transmission from PI calves to cows ranged from 0.006–0.04 new cases per day (Radostits and Littlejohns, 1988).

Continual transmission over the course of 2–3 years, without exposure to a PI animal, however, would seem to require a much longer period of shedding from acutely infected animals than has been reported in the literature (Duffell and Harkness, 1985; Niskanen et al., 2000). If transmission in these herds was in fact due only to shedding by animals with an acute infection, the shedding period for acute infection would have to be considerably longer than the 1–3-week period typically quoted (Duffell and Harkness, 1985; Houe, 1995). The slow transmission in the presence of PI animals in one of the herds also was incompatible with the prevailing dogma about the high, continual risk of transmission from PI animals. One could reason that if a PI animal continuously shed large amounts of virus, all the animals in the herd, regardless of the animal density, would have come in contact with and been infected by a PI animal within 2 years. An alternative explanation for such a slow transmission would be intermittent shedding, either by acutely infected animals or by PI animals, as discussed above. Intermittent shedding by acutely infected or PI animals could explain both the cyclicity in new cases and the slow rate of spread in such small herds.

Under intensive management conditions, such as feedlot operations, in which animal density can be as high as 9.7 animals/100 m² (Rush et al., 2001) and PI animals are invariably present, transmission of BVDV can be much more rapid. For a dry-lot dairy herd practicing MLV BVDV vaccination, 97%

of the heifers showed evidence of infection by the time they reached 13–14 months of age (Michel et al., 1993), and calves entering feedlots seroconverted to BVDV at the rate of 0.4% (Taylor et al., 1995) to 0.7% per day (Martin and Bohac, 1986). The highest incidence of seroconversion on two large dry-lot dairy calf operations was 0.5% and 1.3% per day for 7-month-old heifers (Rush et al., 2001). In another study, when a PI animal was yarded with susceptible cows in that same herd, the incidence rate increased from 0.006–0.04 new cases per day to 0.6 new cases in less than 12 hrs (Radošits and Littlejohns, 1988).

INTER-HERD TRANSMISSION

Considerable opportunity exists for transmission of BVDV from herd to herd, depending on the specific herd biosecurity policies, as reviewed elsewhere (Harkness, 1987). Introduction of new animals into a herd was found to be the main factor significantly associated with high herd seroprevalence of BVDV (Mainar-Jaime et al., 2001). Use of semen from an infected bull also was observed to spread the virus to a number of closed, BVDV-negative herds (Kirkland et al., 1994). Other means of inter-herd transmission include cross-fence contact with infectious animals, their secretions, or excretions; embryo transfer involving introduction of pregnant recipients carrying a fetus with PI, even if the recipient is uninfected; use of MLV BVDV vaccines; and feeding calves waste or “hospital” milk from dairies with lactating PI cows. Ruminants other than cattle can become infected with BVDV (Nettleton, 1990); however, the risk of transmission from these animals is not known.

MINIMIZING TRANSMISSION OF BVDV

REDUCING THE COEFFICIENT OF INFECTIOUSNESS (β)

One strategy in reducing transmission is to reduce the infectiousness of infected animals. The probability that an infected animal will transmit the infection to a susceptible animal may be reduced, under some conditions, by maximizing the SN antibody titer, either through colostral antibodies or vaccination. As discussed above, animals with SN antibodies at the time they acquire an acute BVDV infection may be expected to shed less virus, and for a shorter period of time, than if they had no antibodies. The fact that viremia in PI animals can cease in the presence of SN antibodies (Howard et al., 1989; Palfi et al.,

1993; Brock et al., 1998; Grooms et al., 2001) suggests that shedding also might be diminished following vaccination or ingestion of colostral antibodies. Certainly the main means of diminishing the overall herd infectiousness would be by removal of PI animals because PI animals shed considerably more virus than acutely infected animals.

REDUCING THE LIKELIHOOD OF ADEQUATE CONTACT

As shown by the equations for R_0 and incidence of BVDV infection, the rate of adequate animal-to-animal contact is very important in fostering transmission. In a prospective study (Rush et al., 2001), transmission was related not only to animal density but also to the absolute number of animals in a pen or corral (stocking rate). A small number of animals in a high-density corral or pasture would likely experience a lower risk of infection than a large number of animals in a low-density corral because a corral with many animals is more likely to contain a PI animal, regardless of the density. Strategies for control of BVDV transmission include limiting contact between animals by housing calves in individual isolation hutches (Rush et al., 2001), decreasing density, increasing feed bunk space and water troughs, reducing access to cross-fence sharing of feed and water troughs, double fencing to reduce cross-fence physical contact, and reducing the number of animals in a pasture or pen.

REDUCING THE DURATION OR PREVALENCE OF INFECTIOUS ANIMALS

Duration of infectiousness may be reduced in acutely and perhaps in persistently infected animals by providing adequate SN antibody, as discussed above, for reducing the coefficient of infectiousness. A fundamental strategy in controlling transmission of BVDV is to identify and remove PI animals, thereby reducing the prevalence of infectious animals in a herd.

REDUCING THE PROPORTION OF SUSCEPTIBLE ANIMALS

Because transmission cannot proceed without susceptible animals, a key concept in herd-based control of BVDV transmission is to maximize individual and herd immunity through high-quality colostrum management and strategic vaccination to maximize the repertoire of SN antibody diversity, which are aimed at reducing the probability of animals acquiring a BVDV infection at critical periods when BVDV infection could have important nega-

tive consequences. Simulation modeling suggests that as herd immunity to BVDV wanes, and more and more animals become susceptible to infection, the number of PI animals will increase (Innocent et al. 1997).

SUMMARY

In summary, BVDV can be transmitted briefly by animals following an acute infection and continuously by animals with a persistent BVDV infection. Although presumably rare, it is not known the extent to which virus shedding can be reactivated in acutely infected animals some time after an initial infectious period has waned or by which shedding can be intermittent in PI animals. Animals in the infectious phase of a BVDV infection shed less virus than PI animals, and R_0 values for transmission from acutely infected animals typically can be expected to be in the order of magnitude of <5 , whereas those for transmission from PI animals generally would be >10 – 20 . Consequently, vaccine efficacy and/or the proportion of animals immunized by vaccination must be higher to offer protection against infection acquired from PI animals than from acutely infected animals. Vaccine efficacy aimed at preventing infection is correlated with the degree of homology between the strain of virus used in the vaccine and the strain of virus being prevented. Vaccination may be ineffective in herds with field strains sufficiently heterologous to the vaccine strain to prevent development of adequate protecting cross-reacting SN antibodies. Vaccines employing only one genotype of the virus should not be expected to be efficacious in preventing infection with a strain of the other genotype.

As with other infectious diseases, the forces driving transmission of BVDV in a susceptible population are the infectiousness of the virus (coefficient of infectiousness = β), the contact rate between infectious and susceptible animals, the duration of infectiousness (or proportion of the population that is infectious), and the proportion of the population that is susceptible to infection. Each of these forces should be addressed in programs to limit or control BVDV infection. Vaccination is aimed generally at slowing or stopping transmission by promoting herd immunity; specifically, this is accomplished by reducing the proportion of susceptible animals (increasing the proportion that are immune), and by shortening the shedding period (reducing the duration of infectiousness) and lowering the amount of virus shed (reducing β) for animals that do become infected.

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6

Clinical Features

James F. Evermann and George M. Barrington

INTRODUCTION

Understanding the clinical manifestations of bovine viral diarrhea virus (BVDV) has been a continual challenge for the veterinarian (Tremblay, 1996). Grooms et al. (2002) described five forms of clinical BVDV: acute BVDV infection, severe acute BVDV infection, hemorrhagic BVDV infection, acute BVDV infection–bovine respiratory disease (BRD), and acute BVDV infection–immunosuppression. A significant percentage (70–90%) of BVDV infections results in subclinical infections. However, whether the BVDV infection is one of the acute forms or is subclinical in nature, there is a certain period of virus shedding. These transiently infected (TI) cattle can be a source of virus to susceptible ruminants in the population (Cherry et al., 1998). If the susceptible animal is pregnant, there is a risk of fetal exposure resulting in early embryonic death, abortion, or congenital infections/defects (Mickelsen and Evermann, 1994; Muñoz-Zanzi et al., 2003). An important consequence of congenital infection results from infection of the fetus between 90 and 120 days of gestation. Infection of naive cows during this period results in the birth of calves, which are immunologically vulnerable to BVDV-specific induced tolerance (McClurkin et al., 1984). This tolerance results in a condition commonly referred to as *persistent infection (PI)*. The hallmark of the BVDV PI is that while the animal may appear clinically normal, it has a prolonged viremia (in most cases lifelong) during which it sheds large quantities of virus (Figure 6.1; Bolin, 1995).

This chapter focuses primarily on the clinical outcome of BVD viral infection, and in the process, poses key questions on the ecology and epidemiology of BVDV. These questions will include the following:

- Is the clinical presentation of BVDV infection changing as new strains of virus emerge?
- Is the acute (transient) or chronic (persistent) phase of BVDV infection altered due to the use of BVDV vaccination?
- Is fetal infection with BVDV increasing or decreasing?

We also review the course of initial BVDV infection in naive immunocompetent cattle, recognition of clinical symptoms associated with acute and persistent BVDV infections, duration and severity of clinical symptoms, and recovery from subclinical and clinical symptoms.

An important aspect of BVDV infection is interspecies transmission and the possible spread of BVDV strains among small and large domestic ruminants, as well as wildlife species such as deer, elk, and moose (Loken, 1995; Nettleton and Entrican, 1995). This topic concludes with the feasibility of BVDV control in populations, and whether eradication is achievable at different levels of livestock production.

COURSE OF INITIAL INFECTION IN NAIVE IMMUNOCOMPETENT CATTLE POPULATION

Infection of cattle with BVDV can result in a wide spectrum of clinical manifestations, from imperceptible subclinical infections to fulminant signs ending in death (Baker, 1995). The clinical response to infection is complex and depends on several host and agent factors (Ames, 1986). Host factors that influence the outcome of clinical disease include immunocompetence, immunotolerance, pregnancy status, gestational age of the fetus, the immune status (passive or active immunity from previous infection or vaccination), and the level of environmental stress (Baker, 1995). Viral factors influencing clinical outcome include genomic and antigenic diversity among BVDV isolates (Archambault et al., 2000;

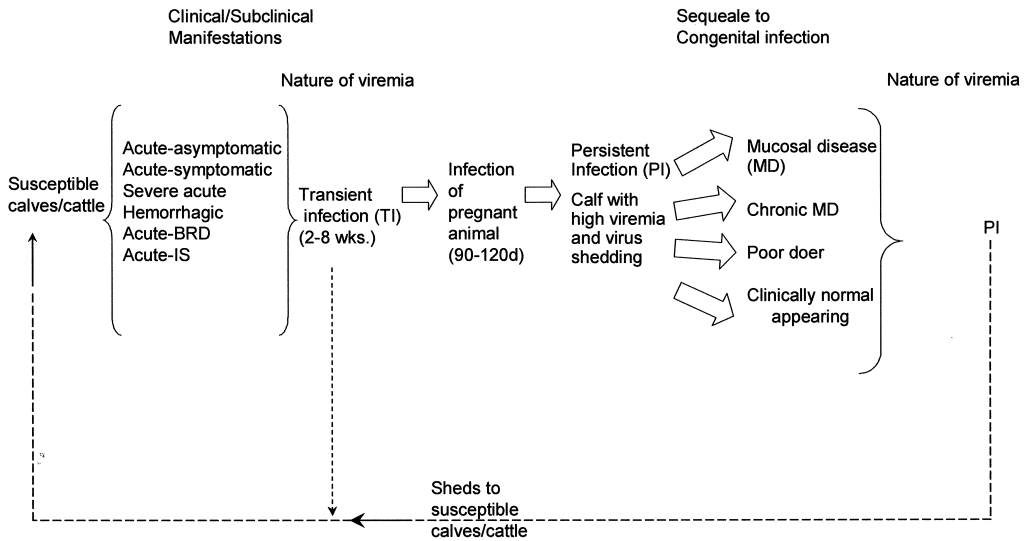


Figure 6.1. Schematic depicting the clinical and subclinical manifestations of BVDV infections and sequelae to congenital infection with BVDV. Abbreviations used are bovine respiratory disease (BRD), immunosuppression (IS), transient infection (TI), and persistent infection (PI).

Bolin and Ridpath, 1996; Ridpath et al., 2000). Currently, two distinct genotypes (BVDV type 1 and BVDV type 2), as well as 11 subgenotypes (BVDV types 1a–1k) are recognized (Brownlie et al., 2000; Vilcek et al., 2001). Differences in virulence among isolates have been recognized for over a decade (Bolin and Ridpath, 1992). Interestingly, recent epidemiologic studies have revealed that the incidence of infection with specific genotypes, as well as the clinical outcome after infection, have changed over time (Evermann and Ridpath, 2002).

Bovine viral diarrhea virus is acquired primarily through aerosols that infect nasal mucosa. The aerosols may become suspended in ambient air and retain infectivity for distances as short as 1.5 m and as far as 10 m (Niskanen and Lindberg, 2003). Direct nose-to-nose contact between an infected animal and a susceptible animal is considered to be the most effective route for BVDV transmission. There are reports of indirect spread of BVDV by use of nose tongs and housing animals in recently (within 2 hrs) contaminated pens (Niskanen and Lindberg, 2003). The virus replicates in draining lymph nodes and from there spreads via circulating lymphoid cells to the blood (Figure 6.2). Viremia can be detected as early as 24 hours postinfection (Mills and Luginbuhl, 1968), and this viremic phase is closely paralleled by the presence of virus in urine 48 hours postinfection. The course of the viremia and the presence of virus in the urine are dependent upon

the presence or absence of colostral antibodies. Colostral BVDV antibody is believed to persist for up to 6 months of age, which accounts for the higher incidence of BVDV-associated disease after this time period (Baker, 1995; Bolin, 1995).

BVDV IN COLOSTRUM-DEPRIVED CALVES

In experimentally infected, colostrum-deprived calves there were diphasic pyrexia, leukopenia, anorexia, and various degrees of diarrhea (Mills and Luginbuhl, 1968). Infectious BVDV was isolated from the spleen and thymus for at least 25 days post-infection (dpi). Lymph nodes draining the forestomachs and intestines were positive for virus until 39 dpi, while those of the duodenum were positive up to 56 dpi. Virus was also isolated from the trachea, lungs, and bronchial lymph nodes at 56 dpi. On the basis of these studies, it was concluded that BVDV is carried in the respiratory tract and may constitute the single most important epizootiologic factor in the pathogenesis of the disease (Mills and Luginbuhl, 1968). These prophetic words were supported repeatedly in subsequent reports on the clinical descriptions of BVDV (Callan and Garry, 2002; Grooms, 1999; Potgieter et al., 1984a).

BVDV IN 6-MONTH-OLD SERONEGATIVE CALVES

A later study using 6-month-old calves reported clinical symptoms limited to a mild bilateral serous nasal

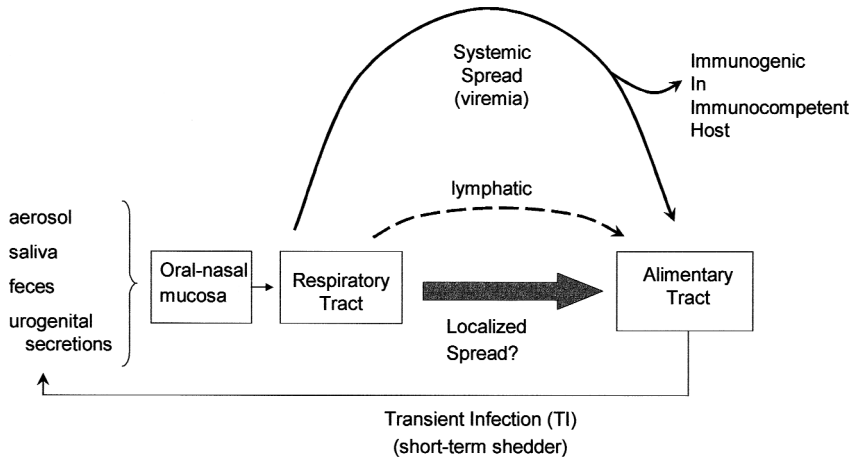


Figure 6.2. Schematic depicting the spread of BVDV in secretions and excretions during a transient infection (TI). Three modes of virus dispersal within the animal's body are noted. These include lymphatics, viremia, and localized spread via the alimentary tract.

discharge at 12 dpi (Wilhelmsen et al., 1990). Although clinical signs observed with the BVDV strain used were minimal, there were significant, immunosuppressive effects of BVDV infection. A 25% or greater decrease in leukocyte count was observed that lasted up to 12 days. In 6-month-old calves there was more evidence of lymphoid involvement of the gastrointestinal tract than in the 2-month-old colostrum-deprived calves. There was gross swelling and edema of mesenteric lymph nodes and lymphocytolysis and edema in the gut-associated lymphoid tissue. This makes it clearer that the following comments are a continuation of the proceeding discussion. Differences noted between BVDV infection of 2-month-old and 6-month-old calves may have resulted from age differences, BVDV strain variation, or a combination of both (Pellerin et al., 1994). Nonetheless, it is apparent that BVDV is capable of multiple systemic effects resulting in different clinical manifestations. Grooms et al. (2002), appropriately wrote about the “diseases” caused by BVDV and indicated that there were at least six initial forms of BVDV infection in nonpregnant cattle and at least two in pregnant cattle (Table 6.1).

BVDV IN PREGNANT CATTLE

The effects of BVDV on pregnant cattle are listed in Table 6.1. Early embryonic death and abortion may be seen following BVDV infection of naive heifers and cows (Bolin, 1990b). Usually up to 85% of cattle become infected, undergo a transient viremia, and seroconvert prior to breeding age. However, this

leaves approximately 15% of the population at risk at the time of initial breeding. It is estimated that BVDV causes about 6–10% of infectious abortions in cattle (Figure 6.3; Dubovi, 1994). Although this infection may result in serious reproductive losses in a few herds, the predominant form of infection with BVDV is the congenital infection (Muñoz-Zanzi et al., 2003). The fetus is highly susceptible between 45 and 125 days of gestation (Dubovi, 1994). Calves infected during this time are at high risk of developing fetal abnormalities that may affect the brain (cerebellar hypoplasia), eyes (retinal atrophy, cataracts), growth retardation with arrested bone development, and pulmonary hypoplasia. Another form of congenital defect is immune tolerance, in which the developing fetus becomes infected with a noncytopathic strain (see Chapter 3) of BVDV between 90 and 120 days (Figure 6.4). The immune system develops tolerance to the infecting strain of BVDV. The result is that no immune response to the virus is mounted by the fetal immune system and the fetus becomes persistently viremic or persistently infected (PI). Figure 6.1 depicts the clinical manifestations of BVDV, with particular attention on the propagation of BVDV PI offspring (see Chapter 7). Following birth, the PI calf is vulnerable to developing mucosal disease (MD) by one of several mechanisms (Figure 6.5).

The impact of different BVDV strains on reproductive performance, in particular age predilection of the fetus for infection with different BVDV genotypes and subgenotypes, was recently reported

Table 6.1. Subclinical and clinical manifestations of bovine viral diarrhea virus (BVDV)*

Category	Manifestations
Acute—Nonpregnant	
Acute/asymptomatic	Mild fever, leukopenia, seroconversion to BVDV, short-term viremia, short-term shedding.
Acute/symptomatic	Fever, leukopenia, depression, anorexia ocular-nasal discharge, oral lesions, diarrhea, decreased milk production in lactating cattle, short-term viremia (15 days), short-term shedding.
Severe acute	Peracute course, fever, pneumonia, sudden death (10–25% mortality). Associated with BVDV type 2.
Hemorrhagic	Marked thrombocytopenia, bloody diarrhea, epistaxis, hyphema, bleeding from injection and branding sites, pyrexia, leukopenia, and death. Associated with BVDV type 2.
Acute/bovine respiratory distress	Fever, pneumonia, anorexia, prolonged treatment, and leading cause of death in feedlot cattle. Secondary infections with <i>Pasteurella multocida</i> , <i>Mannheimia hemolytica</i> , and <i>Mycoplasma bovis</i> complicate the pathogenesis.
Acute/immunosuppressive	Leukocyte function diminished up to 25%, leukopenia, decreased CD+4 and CD+8 T lymphocytes, and decreased macrophage and neutrophil functions. Secondary or polymicrobial infections with bovine respiratory syncytial virus, papular stomatitis virus, malignant catarrhal fever virus have been reported.
Acute—Pregnant	
Abortion	Naive cattle with no prior exposure to BVDV become infected as in acute/asymptomatic or acute/symptomatic above. Viremia leads to placental infection. Abortion, early embryonic death may occur from placentitis. If infection of dam occurs during 90–150 days gestation, congenital infection (CI) of fetus occurs. One form of CI between 90–120 days may result in fetal tolerance to BVDV.
Congenital defects	Infection of a naive dam between 90 and 150 days gestation may result in viremia, which infects fetus resulting in congenital infection (CI). One particular CI occurs between day 90 and 125 and may result in fetal tolerance to BVDV. Calves born with this form of CI are referred to as persistently infected (PI). Calves that are PI have long-term (lifelong) viremia and are long-term shedders of virus, and are at high risk to develop mucosal disease (MD)
Acute/Chronic Sequel to CI	
Mucosal disease	Calves (6–18 months) that are PI are immunotolerant to noncytopathogenic (ncp) BVDV (see Ridpath, Chapter 3). These PI calves may appear clinically normal, but upon superinfection with a homologous BVDV, or mutation of the ncp virus to a cytopathic (cp) variant, or vaccination with a MLV BVDV strain homologous to the ncp PI virus, have a high percentage of developing one of two reported forms of MD—acute or chronic.

Category	Manifestations
<i>Acute/Chronic Sequel to CI</i>	
Mucosal disease (<i>continued</i>)	<p><i>Acute MD</i> is characterized by onset of clinical symptoms within 10–14 days postinfection. Symptoms include biphasic fever, anorexia, tachycardia, polypnea, decreased milk production, and watery diarrhea. Other clinical signs may include nasal-ocular discharge, corneal opacity, excessive salivation, decreased rumination, and bloat. Cattle with acute MD become progressively dehydrated and usually die within 3–10 days. Although the mortality usually approaches 100%, a few animals may survive the acute MD, but are prone to develop chronic MD.</p> <p><i>Chronic MD</i> is a sequel of acute MD. Affected cattle are unthrifty, and may have intermittent diarrhea, chronic bloat, decreased appetite, and weight loss. Nasal-ocular discharge is commonly reported. Cattle with chronic MD rarely survive past 18 months and are usually culled due to low performance or die of severe debilitation.</p>

*Modified from Grooms et al., (2002).

(Evermann and Ridpath, 2002). The results of this study (Table 6.2) indicated that later fetal infections leading to congenital defects and PI calves were associated more with BVDV types 1a and 1b than with BVDV type 2.

RECOGNITION OF CLINICAL SIGNS

SUBCLINICAL INFECTION

It has been estimated that 70–90% of BVDV infections in immunocompetent, seronegative cattle occur without manifestation of clinical signs (Ames, 1986). In closely monitored animals, infection typically results in mild fever, leukopenia, and the development of serum-neutralizing antibodies. In dairy cattle, a decrease in milk production has been associated with subclinical infections (Moerman et al., 1994). Viral replication appears to occur in the upper

respiratory tract and adjacent lymphoid tissues (Bolin, 1990a; Meehan et al., 1998). Subclinical infections appear to account for high levels of serum-neutralizing antibody titers found in most unvaccinated cattle. However, it should be noted that subclinical disease in the pregnant dam might not reflect the severity of the effects of viral infection to the exposed fetus.

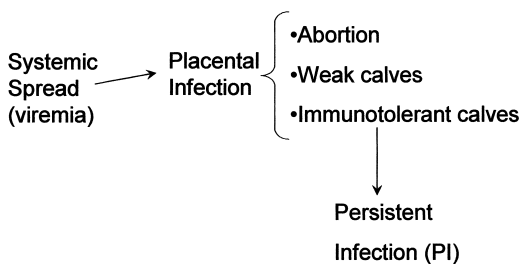


Figure 6.3. Schematic depicting the origin of persistently infected (PI) calves following viremia and placental infection.

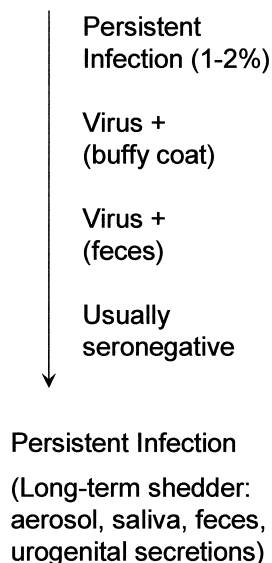


Figure 6.4. Schematic depicting the status of samples from persistently infected calves and the occurrence of virus in plasma/white blood cells (buffy coat) and feces from affected animals.

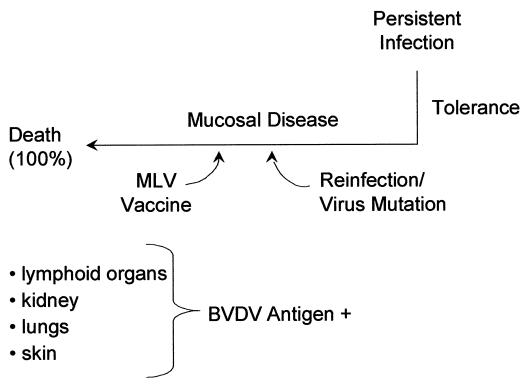


Figure 6.5. Schematic depicting the outcome of a persistently infected calf, which is vulnerable to mucosal disease upon reinfection with homologous BVDV, mutation of tolerizing noncytopathogenic BVDV to cytopathogenic, or vaccination with a homologous strain of BVDV.

CLINICAL BVDV INFECTION

Acute BVDV infection is the term used to describe clinical disease that occurs in non-persistently infected, immunocompetent cattle. The disease has typically been described in seronegative cattle from 6–24 months of age. Conceivably, the disease could occur in seropositive cattle that are infected with a viral strain that is heterologous to the one that has caused seroconversion. Clinical signs include varying levels of fever, anorexia, lethargy, leukopenia, ocular and nasal discharge, oral erosions and ulcers, oral papilla blunting and hemorrhage, diarrhea, and decreased milk production in lactating cows. Epithelial erosions may be present in the interdigital space, coronet, teats, or vulva. Tachypnea may be incorrectly correlated with pneumonia, but is most likely due to fever or other nonpulmonary factors.

Acute BVDV infection of neonates may result in signs of enteritis or pneumonia. Such signs are thought to be most commonly associated with calves suffering from failure of passive transfer, since passively acquired maternal antibodies are thought to be protective. Clinical disease in the face of adequate passive transfer may be related to antigenic diversity between infecting viral strains and viral strains against which passive immunity was developed. Lastly, inadequate passive immunity combined with immunosuppressive effects of BVDV infection may result in secondary diseases affecting various organ systems.

Acute BVDV infection results in damage to ep-

Table 6.2. Summary of bovine viral diarrhea virus isolates and analysis of genotype compared with clinical presentation

<i>Clinical Presentation</i>	BVDV Genotype* and Subgenotypes		
	<i>1a</i>	<i>1b</i>	<i>2</i>
Early fetal infection (abortion)	14%	22%	45%
Later fetal infection (includes animals that are persistently infected, weak calves, chronic poor doers, and mucosal disease)	86%	78%	55%

*From Evermann and Ridpath, 2002.

ithelial surfaces of the gastrointestinal, integumentary, and respiratory systems (Blowey and Weaver, 2003). Viral antigen has been identified in epithelial surfaces of the tongue, esophagus, intestine, bronchi, and skin, as well as phagocytic cells in lymph nodes, thymus, Peyer’s patches, tonsils, and spleen (Ohmann, 1983). Tonsils and respiratory tract tissues are infected first and from there dissemination occurs to other epithelial surfaces and lymphoid tissues. Virus is retained within mononuclear phagocytic cells of the lymphoid tissue (Ohmann, 1983).

Differential diagnoses for diarrheal diseases of adult cattle include Salmonellosis, winter dysentery, Johne’s disease, gastrointestinal parasites, malignant catarrhal fever, arsenic poisoning, Mycotoxicosis, and copper deficiency (Blowey and Weaver, 2003; Kahrs, 2001). Differential diagnoses for diseases causing oral lesions in cattle include malignant catarrhal fever, blue tongue, vesicular stomatitis, papular stomatitis, foot and mouth disease, and Rinderpest. Differential diagnoses for diseases causing diarrhea in neonatal calves include rota- and coronavirus infections, cryptosporidiosis, *E. coli* infection, Salmonellosis and coccidiosis. Differential diagnoses for respiratory diseases of calves include infection with bovine respiratory syncytial virus (BRSV), *Pasteurella* spp., *Mannheimia* spp., *Hemophilus* spp., *Salmonella* spp., and *Mycoplasma* spp.

SEVERE ACUTE BVDV INFECTION

In the early 1990s, an atypical and significantly more severe form of BVDV infection was recognized in the United States and Canadian cattle populations (Corapi et al., 1990; Pellerin et al., 1994;

Carman et al., 1998). A peracute disease course was described with high morbidity and mortality in all age groups of cattle. In the Quebec outbreak approximately 25% of veal calves died, and in Ontario, all age groups of cattle suffered fever, pneumonia, and sudden death (Pellerin et al., 1994; Carman et al., 1998). Severity varied between herds, though some herds experienced 10–20% mortality. Abortions were also a common occurrence. The gross lesions found in these animals were severe, resembling those of mucosal disease. The viral isolates from these outbreaks were characterized by nucleotide sequencing and compared to more classical BVDV outbreak strains (Ridpath et al., 1994). The novel group of BVDV was designated BVDV type 2, compared to the classical group of viruses, now known as BVDV type 1.

Importantly, not all BVDV type 2 outbreaks are necessarily associated with severe disease. Therefore, a severe outbreak of disease should not be assumed to have been caused by BVDV type 2. Alternatively, it is likely that some BVDV type 1 isolates may be capable of causing severe disease.

Hemorrhagic syndrome is a form of severe acute BVDV that appears to be associated with a noncytotoxic, type 2 strain of BVDV. Affected cattle often suffer marked thrombocytopenia, petechiation, and ecchymoses of mucosal surfaces, epistaxis, bloody diarrhea, bleeding from injection sites or trauma, fever, leukopenia, and death (Corapi et al., 1990) (Figures 6.6 and 6.7). Importantly, during an outbreak variation exists between animals in the expression of clinical signs, and only a minority of animals develop fulminant hemorrhagic syndrome. Others may simply suffer marked lymphopenia and thrombocytopenia and succumb without overt clinical manifestation of hemorrhage. Therefore, during an outbreak of severe acute BVDV, practitioners and producers typically observe a diverse clinical picture. Whereas all cattle with hemorrhagic syndrome suffer from severe acute BVDV, not all cattle with severe acute BVDV necessarily progress to hemorrhagic syndrome. The pathophysiologic mechanism of thrombocytopenia and the hemorrhagic syndrome is not clearly understood. Viral antigen has been shown to be associated with both platelets and megakaryocytes, and in addition to thrombocytopenia, platelet function is altered (Walz et al., 1999). Common differential diagnoses for hemorrhagic syndrome include septicemia with concurrent disseminated intravascular coagulation, bracken fern toxicity, sweet clover poisoning, and anticoagulant toxicity.



Figure 6.6. Petechiation of ocular mucous membranes observed in thrombocytopenic calf.



Figure 6.7. Excessive hemorrhage in a thrombocytopenic calf several hours after being administered a subcutaneous injection (courtesy of Dr. Sheila McGuirk, University of Wisconsin).

MUCOSAL DISEASE

Typically mucosal disease is a sporadic condition wherein <5% of herds are affected. In outbreaks, it is common that multiple animals of similar age are affected because the infection is initiated in the fetus

of cows that are at a similar stage of gestation. During such epizootics, up to 25% of a herd may be infected (refer to Figure 6.7).

An incubation period of 7–14 days has been suggested for MD (Grooms et al., 2002). This time frame is primarily based on experimental infections where a homologous, cp BVDV strain was administered to ncp BVDV persistently infected calves, or such calves were administered a modified live vaccine. Since the majority of cases of MD are thought to occur when cp BVDV mutates *de novo* from cattle persistently infected with a ncp strain, such an incubation time frame may not be pertinent (Figure 6.8).

Clinical signs of acute MD include fever, anorexia, tachycardia, polypnea, decreased milk production, and profuse watery diarrhea. Diarrhea is often characterized by the presence of mucosal shreds, fibrinous casts, blood, and foul odor. Other signs similar to acute BVDV infection may also be present antemortem, but are more pronounced. Erosions and ulcers may be present on the tongue, palate, and gingiva. Oral papilla may be blunted and hemorrhagic. Epithelial erosions may also be pronounced in the interdigital regions, coronary bands, teats, vulva, and prepuce. Additional clinical signs often include ocular and nasal discharge, corneal edema, hypersalivation, decreased ruminal contractions, and bloat. Inflammation of the interdigital space and coronary bands may present as lameness and animals may become laminitic. Clinicopathologic findings often include neutropenia (without a left shift) and thrombocytopenia. Secondary bacterial infections may be manifest as pneumonia, mastitis, and metritis. Cattle suffering acute MD gener-

ally have a case fatality rate approaching 100%. However, a minority of animals may survive the acute phase only to suffer signs of chronic MD (see next section). Postmortem findings of cattle suffering acute MD include variable necrotizing ulcers and erosions throughout the gastrointestinal tract (esophagus, rumen, abomasum, duodenum, jejunum, ileum, cecum, and colon). Erosions may also be present in the nares and upper respiratory tract. Peyer's patches in the small bowel are often necrotic and hemorrhagic. Bowel contents are typically watery and fetid. Differential diagnoses for cattle with acute MD include those listed under acute BVDV infection (Figures 6.9, 6.10, 6.11). Although the clinical signs of hemorrhagic syndrome and MD may be similar, animals given supportive therapy may recover from hemorrhagic syndrome. Supportive therapy rarely has any effect in preventing the death of animals suffering from MD.

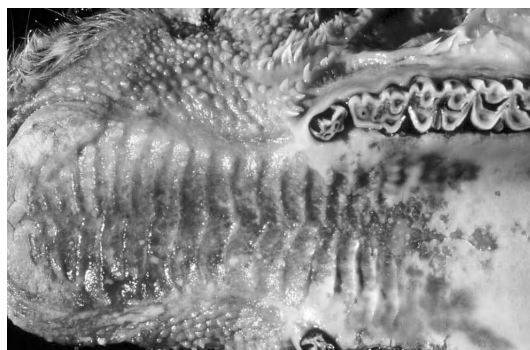


Figure 6.9. Postmortem image of the palate of calf suffering mucosal disease. Note mucosal ulceration and blunting of oral papillae. Similar lesions may be observed in cattle suffering severe acute BVDV infection.



Figure 6.8. Three age-matched beef calves from a herd suffering an outbreak of mucosal disease.

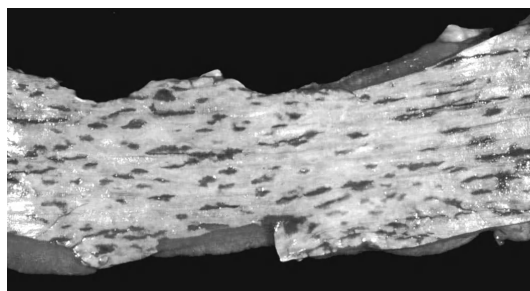


Figure 6.10. Postmortem image of esophageal ulcers and erosions in calf suffering mucosal disease. Similar lesions may be observed in cattle suffering severe acute BVDV infection.

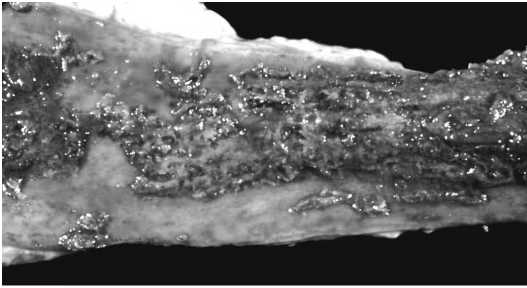


Figure 6.11. Postmortem image of colon lesions in calf suffering mucosal disease. Similar lesions may be observed in cattle suffering severe acute BVDV infection.

CHRONIC MUCOSAL DISEASE

A small proportion of cattle with symptoms of acute MD do not die, but go on to develop a chronic form of the disease. These cattle typically present with persistent loose feces or intermittent diarrhea. Additional signs include ill thrift, mild-to-moderate anorexia, chronic recurrent bloat, interdigital erosions, and nonhealing erosive lesions of the skin. Ocular and nasal discharge may also be noted. Alopecia and areas of hyperkeratinization may develop, especially around the head and neck. Lameness may be manifest as chronic laminitis and abnormal hoof wall growth. Clinicopathologic findings often include anemia, neutropenia and thrombocytopenia.

VENEREAL INFECTIONS

Virus is present in the semen of bulls that are persistently infected with BVDV (Revell et al., 1988). Virus is also present in the semen of immunocompetent, transiently infected bulls undergoing acute infection (Kirkland et al., 1991). Furthermore, recent studies have demonstrated that transiently infected bulls can shed BVDV in semen for prolonged periods (months) after they have become non-viremic (Voges et al., 1998; Givens et al., 2003). Therefore, diagnostic testing of semen is recommended due to the potential for non-viremic bulls to shed BVDV in semen. A recent study demonstrated that the conventional testing method of virus isolation (VI) in semen was less sensitive compared to reverse transcription-nested polymerase chain reaction (RT-nPCR).

Both acceptable and unacceptable semen quality has been reported from bulls that were TI or PI with BVDV. Although results of studies examining the effect of BVDV infection on conception rates have been conflicting, under certain circumstances

BVDV infection is likely associated with transient decreased conception rates (Baker, 1995).

REPRODUCTIVE CONSEQUENCES

Bovine viral diarrhea virus has been associated with several reproductive consequences, including reduced fertility and early embryonic death. All the major organs of the female reproductive system are permissive to BVDV, and the distribution of virus is similar between acutely infected and persistently infected cows (Fray et al., 2000). Although the precise mechanism responsible for reduced fertility is unknown, changes in ovarian function have been suggested. Ovarian hypoplasia has been reported in PI cows (Grooms et al., 1996), and diffuse interstitial ovaritis has been reported 61 days postinfection (Ssentongo et al., 1980). Grooms et al. (1998) demonstrated that the growth rate and diameter of dominant anovulatory and ovulatory follicles were significantly reduced following acute BVDV infection. Additionally, the numbers of subordinate follicles associated with the anovulatory and ovulatory follicle were also reduced following infection. Three possible mechanisms of how BVDV might compromise ovarian function have been suggested (Fray et al., 2000). First, BVDV might adversely affect pituitary gonadotroph function. Second, BVDV-suppressed plasma estrogen levels may adversely affect ovulation and estrus. Last, BVDV-induced general leukopenia may result in deficient ovarian leukocyte populations, because these cells are vital for normal follicular dynamics.

Kafi et al., (2002) demonstrated a detrimental effect of BVDV on in vitro fertilization and early embryo development; however, the mechanisms affecting these processes were not examined. Clearly, further studies are needed to define the effects of BVDV infection on the various hormones and cytokines that influence early reproductive events.

Infection of immunocompetent pregnant cattle can result in clinical manifestations in the dam similar to those described above—i.e., subclinical to severe, acute disease, or hemorrhagic syndrome. However, additional clinical outcomes in pregnant cattle are related to the potential transplacental transfer of virus to the fetus. Experimental studies have shown that BVDV crosses the placenta with near 100% efficiency; therefore, transplacental infection of the fetus should be anticipated (Duffell and Harkness, 1985). Importantly, the absence of clinical signs in the dam does not imply fetal protection or reduce the possibility of transplacental infection. The primary determinant of the outcome of fetal infection is clearly the gestational age of the

fetus, although other host and viral factors may also contribute to the final outcome.

Abortion results from BVDV infection during pregnancy, and the resultant fetal pathology has been recently reviewed (Fray et al., 2000). Infection of the dam during the preimplantation phase (<40 days) of pregnancy can result in a high incidence of embryonic or fetal mortality, whereas infection between 40 and 125 days gestation can result in fetal death, abortion, mummification, birth of PI calves, and to a lesser degree teratogenesis. In the case of fetal death during the first third of gestation, fetal expulsion can vary and occur anywhere from days to months after infection (Kahrs, 2001). Significant rates of abortion (40%) have been reported when cattle are infected at 100 days gestation under experimental conditions (Done et al., 1980); however, lower rates are thought to prevail under field conditions. Fetal infections during midgestation (125–180 days) typically result in a high incidence of congenital abnormalities, which can approach 100% under experimental conditions (Baker, 1995). Fetal infection during the later stages of gestation does not typically result in abortion but is still possible (Bolin, 1990b). Overall, the incidence of abortion is low in immune herds, but can increase dramatically in non-immune herds (Sanderson and Gnad, 2002). Furthermore, outbreaks of acute severe disease associated with BVDV type 2 have been associated with a higher incidence of abortion (Grooms et al., 2002).

Congenital defects result from transplacental infection of the fetus. It has long been known that BVDV can act as a teratogen (Mickelsen and Evermann, 1994). Transplacental infection of the fetus from approximately 100–180 days gestation can be manifest by several congenital defects. During this period, the fetus undergoes the final stages of organogenesis of the nervous system as well as immune system development. The mechanisms of viral-induced cellular damage are thought to include inhibition of cell growth and differentiation, or direct cell lysis (Castrucci et al., 1990). Some common congenital defects include CNS defects (cerebellar hypoplasia, hydrocephalus, hypomyelination), ocular defects (retinal atrophy and dysplasia, cataracts, microphthalmia), thymic hypoplasia, retarded growth, pulmonary hypoplasia, alopecia, hypotrichosis, brachygnathism, arthrogryposis, and other skeletal abnormalities (Baker, 1995).

IMMUNOSUPPRESSION

Acute BVDV infection results in the destruction of lymphoid tissue and immunosuppression (Chase et

al., 2004). Recent studies have examined lesions and tissue distribution of virus in calves experimentally infected with both high- and low-virulence strains of BVDV type 2 (Liebler-Tenorio et al., 2002; Liebler-Tenorio et al., 2003a, 2003b). Widespread lymphoid depletion was noted in calves infected with either strain, and a strong correlation existed between the distribution of viral antigen and the lesions in lymphoid tissue. With the high-virulent BVDV strain, apoptosis and lymphocyte depletion was detected in all compartments where viral antigen was observed (Liebler-Tenorio et al., 2002). With the low-virulent strain, significant lymphoid depletion was rapidly followed by the clearance of viral antigen and subsequent recovery of lesions (Liebler-Tenorio et al., 2003a). Interestingly, viral antigen was not associated with lesions in non-lymphoid tissues (lung, liver, kidney, pancreas, testes, or heart), suggesting that the development of lesions are not solely a function of viral replication but are attributable to the host's reaction to infection. Vaccination of cattle with modified live virus (MLV) vaccines has also been associated with temporary immunosuppression (Potgieter, 1995).

BVDV-induced immunosuppression may increase susceptibility of the host to other pathogens or exacerbate the pathogenicity of co-infecting organisms. Synergistic effects of BVDV infection have been described when animals are concurrently infected with *Mannheimia haemolytica*, bovine herpesvirus type 1, or BRSV. BVDV infections have also been associated with concurrent infection with *Salmonella* spp., *E. coli*, bovine papular stomatitis virus, rotavirus, or coronavirus (Grooms, 1999).

The mechanism of BVDV immunosuppression appears to be multifactorial. Both lymphocytes and macrophages are targets of BVDV infection (Bruschke et al., 1998). Acute BVDV infection can result in transient leukopenia and depletion of lymphoid tissues (Bolin et al., 1985). Decreased B-lymphocytes, CD4+ and CD8+ T-lymphocytes, and neutrophils have been reported (Ellis et al., 1988). In vitro studies revealing potential causes of immunosuppression have been reviewed and include decreased mitogen stimulation of infected lymphocytes, decreased production of interferon, monocyte interleukin 1, interleukin 2, and tumor necrosis factor- α , decreased monocyte chemotaxis, and impaired neutrophil-mediated, antibody-dependant, cell-mediated cytotoxicity (Grooms et al., 2002). Decreased bactericidal activity of neutrophils has also been described (Roth et al., 1981). Finally, BVDV-induced immunosuppression may indirectly

result from prostaglandin release by infected cells (Markham and Ramnaraine, 1985).

BOVINE RESPIRATORY DISEASE

Experimental studies have demonstrated that BVDV can establish infections in the respiratory tract of cattle and that differences in pneumopathogenicity exist between isolates (Potgieter et al., 1985). Despite this finding, the majority of evidence suggests that BVDV infection primarily has a negative impact upon the respiratory defenses, most likely through immunosuppressive effects (Callan and Garry, 2002). Indeed, synergistic effects have been documented between BVDV and *M. haemolytica*, bovine herpesvirus 1, BRSV, and *Mycoplasma bovis* (Potgieter et al., 1984a; Potgieter et al., 1984b; Pollreis et al., 1997; Broderson and Kelling, 1998; Shahriar et al., 2002).

DURATION AND SEVERITY OF CLINICAL SIGNS

As stated earlier, infection of immunocompetent nonpregnant cattle can result in a wide spectrum of disease syndromes progressing from subclinical to severe. The incubation period for acute BVD is 5–7 days (Grooms et al., 2002). Signs of fever, lethargy, anorexia, oral lesions, oculonasal discharge, diarrhea, and decreased milk production in lactating cows follow. Viremia can last up to 15 days. The duration of clinical signs is therefore variable and depends on the duration of viremia, virulence of the infecting virus, the presence of secondary infections, and the normal regenerative capacity of affected tissues. In general, repair of lesions involving epithelial surfaces requires 1–2 weeks, and repair of lesions involving mucosal surfaces of the gastrointestinal tract require approximately 3–5 days. Results from experimental studies suggest that recovery is prolonged with more virulent strains of BVDV since the virus is more widespread in tissues and is more slowly cleared (Liebler-Tenorio et al., 2003b). In the absence of secondary bacterial infections, abatement of clinical signs would correlate to the normal regenerative capacity of the involved tissues.

Secondary infections influence both the duration and severity of clinical diseases associated with BVDV, especially the acute-transient infections. The fact that BVDV is immunosuppressive allows for secondary infections (synonyms include *concurrent infections* and *polymicrobial infections*) to have a profound effect on animal survival (Bolin, 2002). In some cases, such as the sheep-associated malignant

catarrhal fever (ovine herpesvirus-2) in bison, BVDV may actually serve as the secondary infection or as a dual infection (Evermann, unpublished data). There is laboratory and clinical evidence that nutritional deficiencies, such as selenium deficiency, predispose cattle to more severe BVDV acute infections and disease and concurrent bacterial infections, such as *Hemophilus somnus*.

Duration of clinical signs in cattle suffering acute MD is generally 3–10 days, whereby the eventual outcome is the death of the animal. Affected cattle that survive past this expected time frame are considered to develop chronic MD. Cattle with chronic MD typically have an unthrifty appearance with persistent loose stool or intermittent bouts of diarrhea, chronic bloat, decreased appetite, weight loss, interdigital and coronary erosions, and nonhealing skin lesions. Cattle suffering chronic MD rarely survive past 18 months of age and typically die due to debilitation and emaciation. Chronic MD is distinguished from chronically poor-doing PI calves because the latter are typically affected from birth.

RECOVERY FROM CLINICAL SIGNS

Cattle suffering acute BVDV infection without secondary infections generally recover without major complications. Time to recovery is related to the duration of viremia as well as the severity of lesions. In general, recovery can be observed within 2–4 weeks of the onset of signs. Recovery in cattle suffering secondary infections may be prolonged and is dependent on the severity of secondary infections. As stated previously, cattle suffering acute MD do not survive and death occurs approximately 3–10 days after of the onset of signs. Cattle suffering chronic MD typically do not survive beyond 18 months of age (Bolin, 1995).

OCCURRENCE IN OTHER RUMINANTS

Efforts to eradicate BVDV from cattle are hampered for several reasons, including short-term carrier cattle that have transient infections ranging from 2–8 weeks (Cherry et al., 1998), long-term PI carriers that continue to shed virus to herdmates for life (Bolin, 1995; Wittum et al., 2001), strain variation among BVDV isolates evading host immune defenses (Vilcek et al., 2001; Deregt and Loewen, 1995), and the wide host range of BVDV (Evermann et al., 1993; Nettleton and Entrican, 1995). The host range for BVDV and related pestiviruses (see Chapter 10) includes all ungulates belonging to the

order *Artiodactyla*, within which there are 11 species of swine, and up to 173 ruminant species (Van Campen, et al., 2001).

Clinical symptoms in wildlife, such as axis deer, roe deer, and moose, include fever, corneal opacity, and depression. The severity of the disease associated with BVDV appears to depend upon the viral strain, immune competence of the host animal, concurrent viral infection, and/or nutritional deficiencies, such as copper (Van Campen et al., 2001). Domestic animals that can be infected with BVDV include sheep, goats, and members of the camelidae family (camels, llamas, and alpacas) (Van Campen, et al., 2001; Goyal et al., 2002). Clinical symptoms include diarrhea in camels and llamas (Evermann, et al., 1993; Yousif, et al., 2004), abortion, and ill thrift in pregnant llamas (Belknap, et al., 2000), and congenital defects in camel calves (Yousif et al., 2004).

SUMMARY AND CONCLUSIONS

As clinicians and diagnosticians continue to observe and detect the multifaceted effects of BVDV, one must ask, "Is there such a thing as a true subclinical BVDV infection?" Recent epidemiological data would suggest that BVDV poses considerable economic constraints on a herd (Houe, 1999; Innocent et al., 1997; see Chapter 2). These include

- Immunosuppressive effects of the virus on calves postnatally
- Effects of transient infections upon pregnant cows and abortion
- Effects of transient infections upon reproductive age cattle and delayed rebreeding
- Congenital infections leading to calves with congenital defects and growth retardation, etc.
- Congenital infections resulting in PI calves
- Long-term survivability of PI heifers leading to future PI calves, mortality, and replacement costs

The impact that BVDV has on livestock production from both a subclinical and clinical perspective is of major concern (Houe, 1999; Kelling et al., 2000). A combination of rigorous testing for BVDV PI animals (see Chapter 12) and effective vaccination strategies (see Chapter 13) is imperative for the overall health of beef and dairy cattle (Smith and Grotelueschen, 2004).

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7

Pathogenesis

E. M. Liebler-Tenorio

INTRODUCTION

Pathogenesis is defined as “development of disease.” After infection with BVDV, clinical signs of disease are highly variable (Baker, 1995; Nettleton and Entrican, 1995) because of complex interactions between the etiologic agent and the infected host. Genotype (BVDV 1, BVDV 2—also now recognized as two distinct species of virus), biotype (non-cytopathic [ncp], cytopathic [cp]), and virulence of individual BVDV strains are determinants for the outcome of infection on the virus side. Immune competence, immune status, and possibly other factors determine the outcome on the host’s side. Both host and viral factors vary widely causing the broad range of clinical signs and lesions.

For discussion on pathogenesis of BVDV infections, the following syndromes are distinguished: acute BVDV infection, transplacental/intrauterine infection, persistent infection, and mucosal disease (Figures 7.1 and 7.2). Susceptible cattle of all ages may contract a primary, transient BVDV infection termed *acute BVDV infection* irrespective of the clinical course (subclinical, severe acute, or protracted). The transient acute infection causes severe complications if the infected animal is pregnant. BVDV will cross the placenta and infect the fetus causing transplacental/interuterine infection. There are several outcomes of fetal infections; the most important is infection of the fetus leading to the birth of persistently infected (PI) calves. Persistent infection is the prerequisite for the development of fatal mucosal disease later in life. Finally, to close the circle of infection, animals with persistent infection and mucosal disease are the main sources of virus in outbreaks of acute BVDV infections (refer to Figure 7.2).

ACUTE INFECTION

Acute infections of cattle with BVDV develop when susceptible (seronegative), immune-competent cattle become infected with BVDV. Seropositive cattle, dependent on the levels of antibody titers, are usually not susceptible. The virus sources are often PI animals, while horizontal infection from acutely infected cattle is less likely (Traven et al., 1991; Niskanen et al., 2000). Iatrogenic transmission by contaminated instruments, vaccines, or semen has also been described (Coria and McClurkin, 1978; Barkema et al., 2001; Niskanen and Lindberg, 2003).

ROLE OF VIRAL AND HOST FACTORS IN PATHOGENESIS

The most important determinant for the outcome of acute BVDV infection in susceptible animals is the virulence of the individual BVDV strain. Historically, acute BVDV infections have received little attention, because they were predominantly subclinical (Moennig and Plagemann, 1992; Hamers et al., 2001), and the unspecific signs of transient fever and listlessness did not incite high awareness. Until the early 1990s, there were only a few reports about acute BVDV infections causing severe clinical signs or with special affinity for the respiratory tract (Potgieter et al., 1984). Over the last 15 years, however, there has been an increasing interest in acute BVDV infections because of an increase in incidence of field cases of acute BVDV infection associated with severe clinical signs and mortality in all age groups (Perdrizet et al., 1987; Pritchard et al., 1989; Hibberd et al., 1993; David et al., 1994; Carman et al., 1998).

Highly virulent BVDV strains isolated from the outbreaks that had severe economic impact in North

outcomes of infections with BVDV

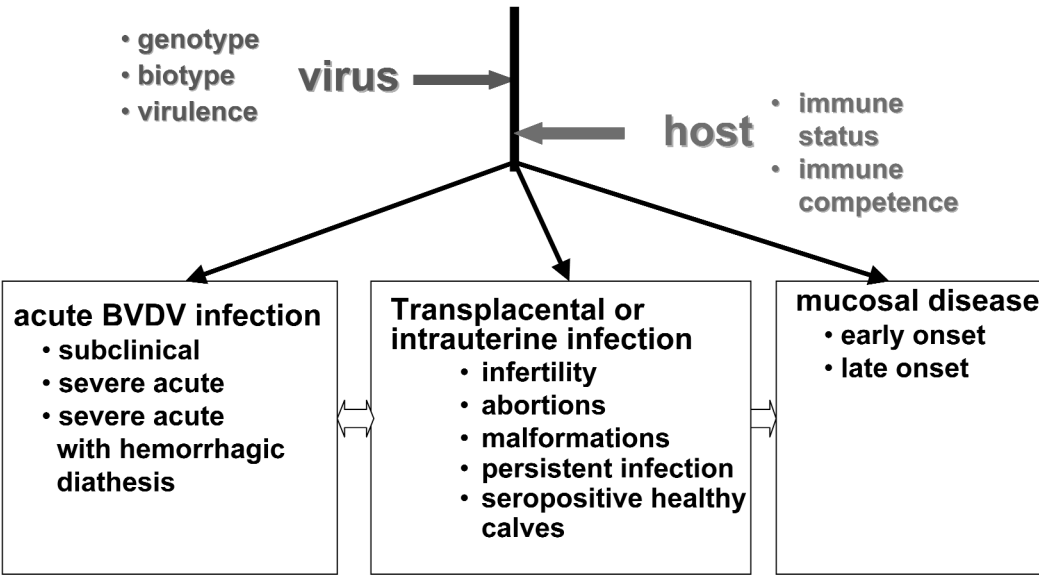


Figure 7.1. Outcomes and factors influencing the outcome of BVDV infections.

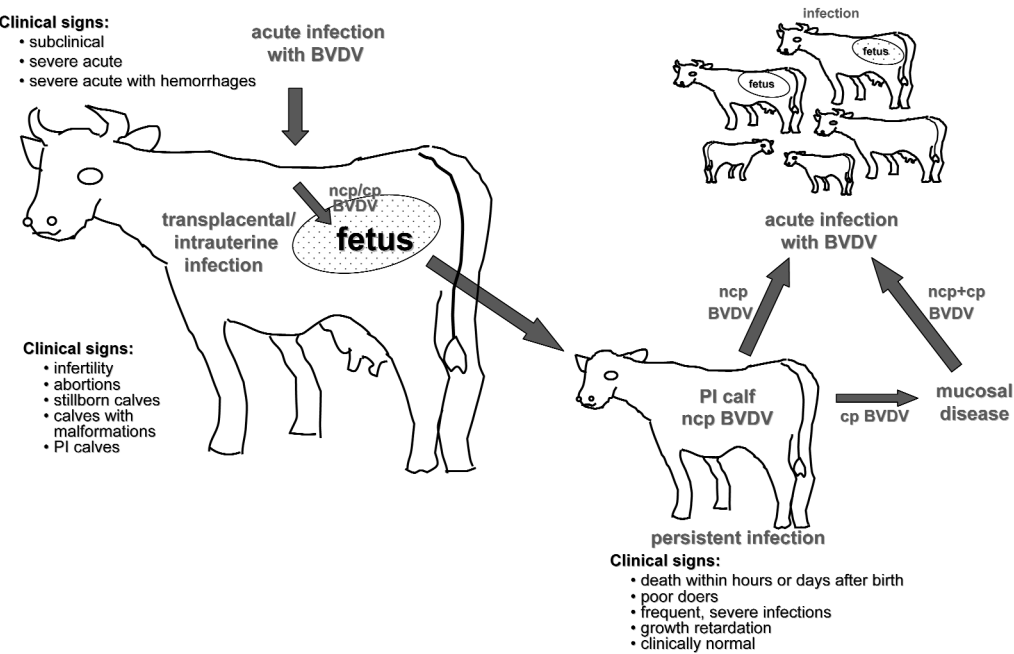


Figure 7.2. Circulation of BVDV in cattle populations.

America were identified as BVDV 2 (Ridpath et al., 1994; Heinz et al., 2000). Further investigation revealed, however, that BVDV 2 encompasses not only strains of high virulence, but also those of moderate to low virulence (Ridpath et al., 2000). Virulence factors associated with BVDV have not yet been defined on an antigenic or genetic level. The occurrence of virulence factors is independent of viral species, although the frequency of virulent strains appears to be higher in BVDV 2 than in BVDV 1. There are regional differences in the relative frequency of BVDV species. Although a high percentage of BVDV strains belong to BVDV 2 in North America (Bolin and Ridpath, 1998; Carman et al., 1998; Fulton et al., 2000), they are infrequently isolated in other countries (Wolfmeyer et al., 1997; Letellier et al., 1999; Sakoda et al., 1999; Tajima et al., 2001; Drew et al., 2002; Vilcek et al., 2002). This is reflected in the different frequency of severe acute BVDV infection in different countries.

Virulence is not correlated with the biotype, since both ncp and cp strains are represented in both BVDV 1 and BVDV 2 and have a wide spectrum of virulence. There are, however, differences between the ncp and cp biotype. ncp BVDV spreads much wider in the host than the homologous cp virus (Spagnuolo-Weaver et al., 1997). The ncp and cp biotype also differ in the way they activate the immune system (Collen and Morrison, 2000). Although ncp BVDV induces a more pronounced humoral response, the response to cp BVDV is skewed toward a cell-mediated immune response. This is particularly evident upon secondary exposure to cp BVDV (Lambot et al., 1997). One can hypothesize that this is mediated by the different effects of ncp and cp BVDV on monocytes and dendritic cells. After *in vitro* infection, monocyte function is altered by ncp BVDV, but they are killed by cp BVDV. Dendritic cells are not affected by infection with either ncp or cp BVDV (Glew et al., 2003).

The highly variable clinical signs present difficulties for diagnosing BVDV infections in the field. Even if acute BVDV infection is suspected, it may be difficult to confirm, since virus is only transiently detectable (Sandvik et al., 1997). Except for immune status, the role of other host factors on BVDV infection has not yet been determined. However, because of differences in clinical signs and speed of recovery between individual animals after experimental infection, the role of host factors should not be neglected (Liebler-Tenorio et al., 2003a).

VIRUS SPREAD AND DEVELOPMENT OF LESIONS IN ACUTE BVDV INFECTION

The most frequent route of natural infection is by oronasal uptake of BVDV. The development of clinical signs and distribution and spread of the virus in the host and tissue lesions have been documented in field cases and under experimental conditions (Lambert et al., 1969; Corapi et al., 1990; Wilhelmsen et al., 1990; Traven et al., 1991; Bolin and Ridpath, 1992; Castrucci et al., 1992; Marshall et al., 1996; Spagnuolo-Weaver et al., 1997; Bruschke et al., 1998a; Ellis et al., 1998; Odeon et al., 1999; Archambault et al., 2000; Hamers et al., 2000; Stoffregen et al., 2000; Liebler-Tenorio et al., 2002, 2003a,b). The data were collected primarily for virulent BVDV strains, but some data do exist for strains of low virulence (Wilhelmsen et al., 1990; Traven et al., 1991; Bolin and Ridpath, 1992; Marshall et al., 1996; Bruschke et al., 1998a; Liebler-Tenorio et al., 2003a). In the following section, the findings for strains of low and high virulence will be described separately.

Strains of low virulence

After intranasal inoculation of calves with BVDV 1 and BVDV 2 strains of low virulence, the animals did not develop overt signs of disease (Wilhelmsen et al., 1990; Traven et al., 1991; Bolin and Ridpath, 1992; Marshall et al., 1996; Bruschke et al., 1998a; Liebler-Tenorio et al., 2003a). The regular monitoring of body temperature showed, however, a mild elevation in temperature for 1–2 days (Liebler-Tenorio et al., 2003a). This elevation was very short-lived, making it necessary to monitor temperature twice a day.

Virus isolation and titration data indicate that BVDV 1 first replicates in the tonsils and nasal mucosa (Bruschke et al., 1998a). Viral antigen was initially found in the tonsils, lymph nodes, and Peyer's patches and then in spleen and thymus (Liebler-Tenorio et al., 2003a) (Figure 7.3). Viral antigen was predominantly present within lymphoid follicles and the thymic cortex where it was associated with lymphocytes and stromal cells. Infection in these sites affected the majority of cells present. Multifocal infection of the intestinal mucosa was the only site outside of lymphoid tissues where viral antigen could be demonstrated (refer to Figure 7.3). Viral antigen was not detectable in the bone marrow at any point during infection. Presence of viral antigen was not associated with tissue lesions.

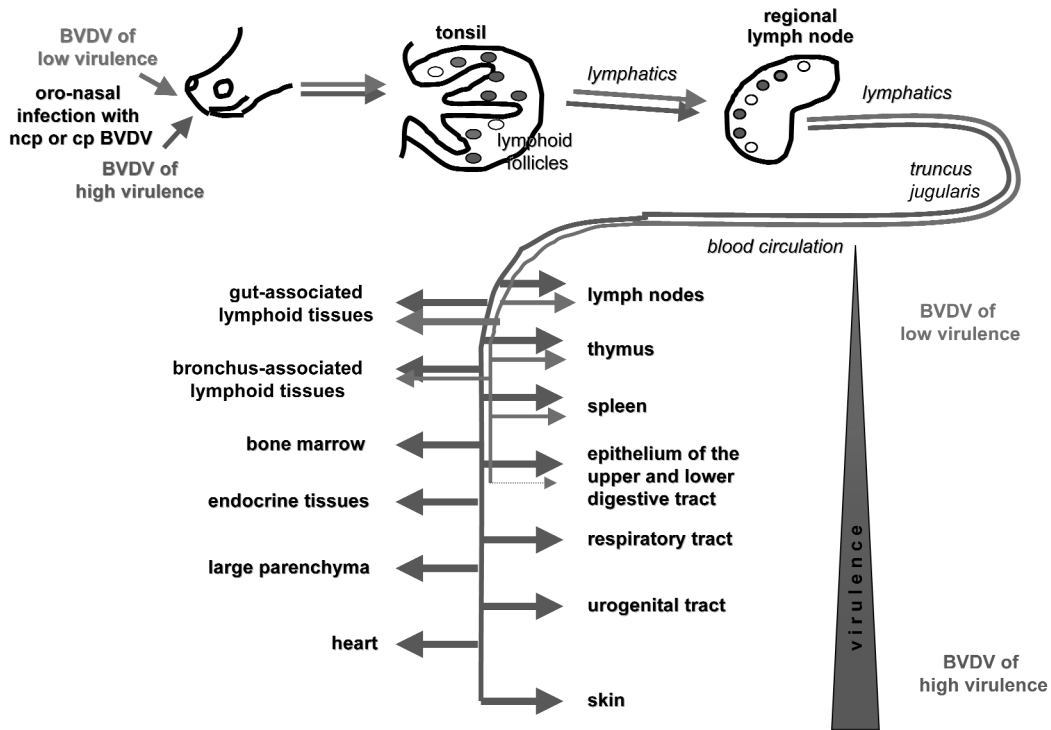


Figure 7.3. Spread of BVDV of low and high virulence in acute BVDV infection.

The widest distribution and the largest amount of viral antigen was found at 6 days postinfection (dpi) followed by a rapid clearance of the virus leading to its disappearance from most sites. The remaining viral antigen was associated with follicular dendritic cells in lymphoid follicles and dendritic cells in the thymus. Viral clearance coincided with the loss of infected lymphocytes leading to a marked depletion of lymphoid follicles and thymic cortex of lymphocytes.

After virus clearance, different degrees of depletion and repopulation were observed in the lymphoid tissues throughout the body. There was a marked difference in the degree of recovery between individual calves indicating the importance of host factors. The reversibility of the lesions was most likely due to the fact that the stromal elements were retained in the lymphoid tissues allowing repopulation with circulating lymphocytes.

Infection with BVDV strains of high virulence

Clinical signs after infection with strains of high virulence are severe but are often nonspecific consisting of high fever, anorexia, depression, and frequently diarrhea (Corapi et al., 1990; Bolin and Rid-

path, 1992; Ellis et al., 1998; Odeon et al., 1999; Archambault et al., 2000; Liebler-Tenorio et al., 2002). Some animals develop severe bleeding (Corapi et al., 1990; Bolin and Ridpath, 1992; Stoffregen et al., 2000). Bleeding is a clinically very dramatic and easy-to-recognize alteration and thus the term *hemorrhagic syndrome* has been associated with severe acute BVDV infections although it does not occur regularly. The mortality rate may be high even in older cattle (Carman et al., 1998). In experimental infections, early onset of high fever is a consistent finding (Ridpath et al., 2000; Liebler-Tenorio et al., 2002). The other consistent findings are severe progressive lymphopenia and moderate to severe thrombocytopenia (Corapi et al., 1990; Bolin and Ridpath, 1992; Marshall et al., 1996; Ellis et al., 1998; Odeon et al., 1999; Archambault et al., 2000; Hamers et al., 2000; Stoffregen et al., 2000; Liebler-Tenorio et al., 2002).

The initial spread of highly virulent strains is similar to that of low virulence strains (Liebler-Tenorio et al., 2002, 2003b) (refer to Figure 7.3). There is initial infection of tonsils and lymphoid tissues, but the amount of viral antigen in tissues rapidly exceeds that caused by low virulence strains. The presence of

highly virulent BVDV is not restricted to follicles in lymphoid tissues but is extended to T-cell-dependent areas. Highly virulent BVDV spreads to the bone marrow. Its presence in myeloid cells and megakaryocytes correlates with a decrease in thrombocyte numbers.

In contrast to BVDV of low virulence, which is cleared from infected tissues, virulent BVDV strains keep spreading (refer to Figure 7.3) resulting in the tissue distribution described for severe acute BVDV (Marshall et al., 1996; Ellis et al., 1998; Odeon et al., 1999; Stoffregen et al., 2000). Antigen of highly virulent BVDV is regularly found in lymphoid tissues, mucosa of the upper and lower digestive tract, the respiratory tract, and endocrine tissues. Eventually, there is hardly any organ that does not contain viral antigen at least in the interstitium. Antigen is often initially present in the interstitium or vascular walls and then in parenchymal cells, indicating a hematogenous spread. Within organs, virus spreads to additional tissue types; e.g., in the intestine where initially the mucosa is antigen-positive, it can later be found in the muscular layers.

Despite the wide distribution of viral antigen, the presence of lesions is restricted. Lesions accompanied by the loss of lymphocytes are consistently observed in lymphoid tissues (Marshall et al., 1996; Ellis et al., 1998; Odeon et al., 1999; Stoffregen et al., 2000; Liebler-Tenorio et al., 2002). Necrosis of epithelium in the upper digestive tract and intestinal crypts may cause erosive to ulcerative lesions along the digestive tract (Marshall et al., 1996; Carman et al., 1998; Ellis et al., 1998; Odeon et al., 1999; Stoffregen et al., 2000).

The discrepancy between presence of viral antigen and lesions is particularly evident in the initial phase of the disease (Liebler-Tenorio et al., 2002). Initially, large numbers of infected cells are present in lymphoid tissues without corresponding morphological lesions. Lesions in lymphoid tissues are delayed. In the thymus, viral antigen is diffusely present in thymic lobules, but lesions are initially multifocal. The same phenomenon can be found in the intestinal mucosa, where viral antigen has a diffuse distribution, but initial lesions can be observed multifocally. In the lungs, frequently foci of acute purulent bronchopneumonia are seen, which do not correlate with the distribution of BVDV antigen in the lung.

Comparison between strains of low and high virulence

Experimental infection with BVDV strains of low and high virulence reveals similar initial infection

and spread but differing amounts of virus in tissues and speed of spreading (Liebler-Tenorio et al., 2003b). This results in a widespread tissue distribution of the virulent strains in later stages of infection, as opposed to an early clearance of the strains of low virulence from tissues. Strains that cause the highest degree of viremia result in the most severe clinical signs (Walz et al., 2001). This indicates that differences in replication, and not interactions between virus and host cell receptors, have the highest impact on the virulence of individual BVDV strains. One can hypothesize that there is a competition between viral spread and reaction of the immune system. The pathogenesis of tissue lesions is unsolved; the discrepancy between the presence of viral antigen and lesions as well as the delayed onset of lesions seen with low and highly virulent BVDV strains might indicate that immune-mediated reactions contribute to the development of lesions.

IMMUNE SUPPRESSION IN ACUTE BVDV INFECTION

A decrease in the number of both B- and T-lymphocytes in peripheral blood is a consistent finding in acute BVDV infection (Bolin et al., 1985a). The severity of lymphopenia helps discriminate infections with low or highly virulent BVDV strains (Ridpath et al., 2000; Liebler-Tenorio et al., 2003b). Although lymphocyte numbers decrease to more than 60% below the baseline levels, sometimes reaching 90% below the baseline levels after infection with highly virulent strains, the decrease does not exceed more than 50% below the baseline values in strains with low virulence. Lymphopenia correlates well with the infection of and lesions in lymphoid tissue, but it is not resolved yet if lesions are directly virus-induced or if the immune response also contributes to their development.

Experimental infection with virus of low virulence demonstrates clearly that even infections that have a subclinical course and would go unnoticed under field conditions will cause a marked, although transient, immune suppression (Liebler-Tenorio et al., 2003a). This explains reports that combined infections with BVDV have a potentiating effect on several pathogens. Coinfection with BVDV increases the severity of rota- and coronavirus infections in young calves (van Opdenbosch et al., 1981; Kelling et al., 2002; Niskanen et al., 2002b) and the severity of IBRV (infectious bovine rhinotracheitis virus) and BRSV (bovine respiratory syncytial virus) infections (Potgieter et al., 1984; Castrucci et al., 1992; Brodersen and Kelling, 1998, 1999). The

immune response to BRSV is delayed (Elvander et al., 1998). The sequential inoculation of calves with BVDV and *Mannheimia haemolytica* increased the severity of lung lesions (Potgieter et al., 1984; Ganheim et al., 2003). After vaccination with modified live vaccines for BVDV, there was a decreased response to *Mycobacterium paratuberculosis* (Thoen and Waite, 1990). BVDV infection exacerbated the effects of infection with *Salmonella* sp. (Wray and Roeder, 1987) and led to impaired clearance of bacteremia (Reggiardo and Kaerberle, 1981).

Besides inducing lymphopenia, BVDV has a wide range of effects on the functions of specific as well as innate immune responses (Peterhans et al., 2003). However, immune suppression is not associated with low interferon response or elevated levels of TGF- β (Charleston et al., 2002). Peripheral lymphocytes are hyporesponsive to mitogens (Muscoplat et al., 1973; Pospisil et al., 1975). Different neutrophil and monocyte functions are impaired (Ketelsen et al., 1979; Roth et al., 1981, Roth and Kaerberle, 1982, 1983; Jensen and Schultz, 1991). Reduction in nonspecific defense has been suggested as the main contribution of BVDV to respiratory tract infections (Potgieter, 1997; Brodersen and Kelling, 1998). A multitude of functional defects are seen in alveolar macrophages from infected calves and those inoculated in vitro with BVDV (Welsh et al., 1995; Liu et al., 1999).

THROMBOCYTOPENIA AND HEMORRHAGES IN SEVERE ACUTE BVDV INFECTIONS

Thrombocytopenia occurs regularly in cases of severe acute BVD although the reduction of platelets does not always result in marked hemorrhages (Rebhun et al. 1989; Corapi et al., 1990; Bolin and Ridpath, 1992; Ellis et al., 1998; Odeon et al., 1999; Archambault et al., 2000; Hamers et al., 2000; Stoffregen et al., 2000; Liebler-Tenorio et al., 2002). The underlying cause of thrombocytopenia is not completely understood. Necrosis of megakaryocytes, reduced production of thrombocytes by megakaryocytes, increased consumption of thrombocytes in the periphery, and functional defects of thrombocytes have all been suggested as contributing factors (Rebhun et al. 1989; Corapi et al., 1990; Walz et al., 1999, 2001). In some cases of acute BVDV infection, BVDV antigen has been demonstrated in bone marrow (Marshall et al., 1996; Spagnuolo et al., 1997). Experimental infection with BVDV strains of different virulence revealed that not all BVDV strains spread to the bone marrow (Liebler-Tenorio et al., 2003b). The development of

thrombocytopenia is directly related to the infection of bone marrow with BVDV. In the bone marrow, BVDV can be detected in all cellular elements including megakaryocytes (Spagnuolo et al., 1997; Walz et al., 1999; Liebler-Tenorio et al., 2002). Megakaryocytes give rise to thrombocytes by pinching off parts of their cytoplasm. Thus, infection of megakaryocytes appears to be crucial in the development of thrombocytopenia. It is unclear how the infection of megakaryocytes induces a reduction in the number of thrombocytes—e.g., whether there is a reduced production of thrombocytes and/or whether the produced thrombocytes are functionally altered. Investigations by Walz et al. (1999) indicate that both mechanisms are involved.

Bleeding (hemorrhagic diathesis) occurs only when thrombocytes have reached very low numbers. Some infections with highly virulent BVDV may cause mortality early in infection. Since the bone marrow is infected later than other lymphohematopoietic tissues, and thrombocytopenia develops after infection of the bone marrow, the death of the animal may occur before hemorrhagic diathesis becomes established. This might explain the variation in frequency of bleeding observed in the field and in experimental cases of severe acute BVDV infections.

TRANSPLACENTAL/INTRAUTERINE INFECTIONS

The main economic impact of BVDV infections is caused by intrauterine infections resulting in reproductive dysfunctions (Ross et al., 1986; Kirkbride, 1992; Dubovi, 1994; McGowan and Kirkland, 1995; Moennig and Liess, 1995; Muñoz et al., 1996; Rufenacht et al., 2001). This is due to the fact that BVDV is able to infect the female and male genital tracts, cross the placenta and infect the fetus (refer to Figure 7.2). Transplacental infection may occur in the course of acute infections shortly before or during pregnancy, venereal infection by contaminated semen, and persistent infection (discussed in this section in detail). Even acute infections without clinical evidence of disease may lead to infection of the genital tract and transplacental infection (McGowan et al., 1993). Therefore, any infection shortly before or during pregnancy has severe consequences on reproductive performance.

ROLE OF VIRAL AND HOST FACTORS IN TRANSPLACENTAL/INTRAUTERINE INFECTION

The outcome of transplacental/intrauterine infection depends primarily on the time of infection during

pregnancy and thus the gestational age of the early conceptus or fetus. Infections shortly before or during the first weeks of pregnancy disturb the hormonal balance, cause reduced conception rates, and have direct effect on the early conceptus. In later pregnancy, the outcome of BVDV infection is influenced by the development of target cells, phase of organogenesis, and development of the fetal immune system (Duffel and Harkness, 1985).

The sequelae of infection are also determined by viral properties, especially the biotype of BVDV. Both biotypes are able to infect genital tissues, cross the placenta, and infect the fetus (Scott et al., 1972; Done et al., 1980; Brownlie et al., 1989; Muñoz et al., 1996; Grooms et al., 1998b, 1998c), but the results of infection differ. Infection with the cp biotype appears to cause more damage in the early conceptus than that with ncp BVDV (Vanroose et al., 1998). Only ncp BVDV is able to induce persistent viremia (Brownlie et al., 1989). It has been suggested that the ability to inhibit the induction of type 1 interferon enables the ncp biotype to induce viral persistence (Charleston et al., 2001).

Transplacental infection has been reported for both BVDV 1 and BVDV 2 (Wittum et al., 2001). Dual infection of the fetus with both BVDV 1 and BVDV 2 has also been demonstrated (Brock and Chase, 2000), although differences in the extent of infection were observed between BVDV 1 and BVDV 2. Since the BVDV 2 strain was of higher virulence than the BVDV 1 strain, there was wider tissue distribution of the BVDV 2 strain suggesting an influence of virulence on the efficiency of virus replication.

INFECTION DURING THE PREOVULATORY PERIOD

Acute infection of cows with BVDV during the preovulatory period may result in viremia at ovulation leading to a decreased conception rate. If conception occurs, the calves are normal, seronegative, and non-viremic at parturition (McGowan et al., 1993). Both ncp and cp BVDV have been demonstrated to infect the ovaries in experimental infections (Grooms et al., 1998b, 1998c). By immunohistochemistry, BVDV can be found in granulosa and stromal cells of ovaries between 8 and 30 days postinfection (Grooms et al., 1998b, McGowan et al., 2003). Histological examination of the ovaries reveal diffuse nonpurulent interstitial inflammation, necrosis of granulosa cells, and necrosis of follicles (Ssentongo et al., 1980; Grooms, et al. 1998c; Fray et al., 2000a; McGowan et al., 2003). Lesions in the

ovaries occur 2–6 days after estrus during the period of seroconversion and may persist for extended time periods (60 days) postinoculation (Ssentongo et al., 1980; Grooms, et al. 1998c, McGowan et al., 2003).

Ovarian lesions are associated with ovarian dysfunctions characterized by decreased secretion of gonadotrophins and sex steroids, particularly progesterone, and absent or reduced preovulatory luteinizing hormone surge (Fray et al., 2000a, 2002; McGowan et al., 2003). The hormonal imbalance leads to decreased growth of ovarian follicles, reduced ovulation rate, reduced numbers of corpora lutea, reduced numbers of collected embryos, and increased numbers of unfertilized ova (Grahm et al., 1984; Grooms et al., 1998a; McGowan et al., 2003). These changes last as long as the lesions persist, at least the first two estrous cycles after infection. This explains the reduced conception rates and the delays in conception (Grooms et al., 1998a).

Ovarian dysfunction is one way that BVDV infections decrease conception rates. Infection of oocytes during infection of ovaries will also impair fertility (Bielanski et al., 1998). The infection of ovaries is multifocal and does not affect all oocytes. Therefore, cows that conceive despite infection produce normal, seronegative, and non-viremic offspring (McGowan et al., 1993). Up to 58% of embryos collected for in vitro fertilization from transiently infected cows were infected with BVDV (Bielanski et al., 1998). Infection of the embryo may result in slightly reduced survival rate and retarded development of the embryo (Archbald et al., 1979; Bielanski and Hare, 1988; Kafi et al., 2002). BVDV can be isolated from retarded embryos (Kirkland et al., 1993).

EXPOSURE OF THE UTERUS TO BVDV AT INSEMINATION

Exposure of the uterus to BVDV during estrus at insemination causes a decreased conception rate in naive cattle, and seropositive cows have normal conception rates (McClurkin et al., 1979; Whitmore et al., 1981; McGowan et al., 1993; Kirkland et al., 1994). This type of exposure might occur when cows are inseminated with semen contaminated with BVDV or contract acute BVDV infection because of animal movement and mixing around the time of insemination. Experimentally, infection can be reproduced by direct intrauterine instillation of BVDV (Whitmore et al., 1981; Grahm et al., 1984).

Semen contaminated with BVDV may originate from persistently infected bulls, since not all PI bulls are infertile or produce semen of inferior quality

(Meyling and Jensen, 1988; Wentink et al., 1989; Kirkland et al., 1994). On the other hand, infected semen may also be produced following transient infection of postpuberal bulls (Kirkland et al., 1991; Voges et al., 1998; Niskanen et al., 2002a; Givens et al., 2003). Even after transient infection and clearance of the virus from other tissues and blood, BVDV can persist in semen for months after exposure (Kirkland et al., 1991; Voges et al., 1998; Niskanen et al., 2002a; Givens et al., 2003). The most productive sites of viral replication in the male genital tract are the seminal vesicles and prostate glands causing excretion in the seminal fluid (Kirkland et al., 1991). Insemination with infected semen will cause transient infection of naive females (Kirkland et al., 1994).

The effect of BVDV on gametes and the early conceptus were investigated. In vitro investigations revealed no immediate effect of ncp and cp BVDV on the maturation of oocytes to blastocysts, although BVDV replicated well in cells around the embryo (Kirkland et al., 1996; Tsuboi and Imada, 1996; Kafi et al., 2002). In vitro fertilization of oocytes in the presence of BVDV resulted in reduced fertilization rates (Kafi et al., 2002). There was no uptake of BVDV by embryos after in vitro exposure (Potter et al., 1984). It has been suggested that zona pellucida protects the embryo against infection with BVDV (Singh et al., 1982). After in vitro removal of zona pellucida, 2- and 4-cell embryos were not susceptible to infection with ncp BVDV (Tsuboi and Imada, 1999) and 8-cell embryos were susceptible to cp BVDV (Vanroose et al., 1998). Even extended exposure of embryos with removed zona pellucida had no negative influence on embryonic survival (Bielanski and Hare, 1988). With progressive development, the embryos became increasingly susceptible to infection (Bak et al., 1992; Vanroose et al., 1998; Tsuboi and Imada, 1999).

FETAL INFECTION

After about 30 days of gestation the fetus is susceptible to transplacental infection. There appears to be an increasing efficiency of infection from conception to day 30 postconception (Kirkland et al., 1993). This corresponds with a progressive increase in the loss of pregnancies observed between days 20 and 77 after artificial insemination. Development of placenta with the formation of placentomes, formation of trophoblasts and development of fetal tissues most likely determine the efficiency of infection (Kirkland et al., 1993).

Experimental infections or vaccine trials using

modified live vaccines have shown that fetal infections may result in abortion, persistent infection, stillborn calves, teratogenic effects, and normal calves born with antibodies to BVDV. Although both biotypes of BVDV can infect the fetus, only ncp BVDV is able to establish persistent infection (Brownlie et al., 1989). Infection with cp BVDV results either in abortion or healthy seropositive calves (Brownlie et al., 1989). The fetus, before it becomes fully immune-competent, is affected severely by intrauterine BVDV infection. The intrauterine infection of an immune-competent fetus results in a transient infection of the fetus comparable to acute BVD. The fetus is able to mount an immune response and the virus is cleared. Calves are clinically normal and non-viremic. The presence of antibodies to BVDV in serum samples collected before colostrum uptake indicates intrauterine exposure to BVDV. Neutralizing precolostral antibodies have been detected in newborn calves after infections from day 90 to the end of gestation (Kendrick 1971; Orban et al., 1983; Liess et al., 1987).

Abortions

It was recognized early that BVDV may induce abortions (Kahrs and Ward, 1967; Casaro et al., 1971; Kendrick, 1971; Done et al., 1980; Duffell et al., 1984). Abortions occur most frequently in the early stages of gestation (less than 125 days of gestation), although abortions in the late phase of gestation have also been described (Duffell et al., 1984; Roeder et al., 1986). The expulsion of the fetus often occurs not immediately after infection but weeks to months later (Duffell et al., 1984). This also explains the occurrence of mummified fetuses (Scott et al., 1973; Done et al., 1980).

The pathogenesis of abortion is not clear. Most investigations have reported unremarkable, nonspecific lesions of the placenta and placentomes of aborted calves (Casaro et al., 1971; Done et al., 1980; Murray, 1991) but Baszler et al. (1995) found a necrotizing placentitis associated with the presence of viral antigen. It has been speculated that the mild placental lesions might allow opportunistic infections to cross the placenta. BVDV antigen was present in numerous tissues of aborted fetuses, and infiltrates of mononuclear cells were found in several tissues including lung and myocardium (Done et al., 1980; Murray, 1991; Baszler et al., 1995). Therefore, damage to the fetus is considered as important initiator for abortion.

Tissues from pregnant cows infected with an ncp BVDV at 85–86 day of gestation were examined for

viral antigen by immunohistology (Fredriksen et al., 1999a). BVDV was present in the fetuses at 14 dpi in the lung and liver and at 18–22 dpi in all other tissues examined (lung, liver, spleen, intestine, and brain). The intercotyledonary fetal membranes and the placentomes contained viral antigen at days 18 and 22 postinoculation. BVDV antigen was not detected in maternal tissues or in the placenta between days 7 and 22 postinoculation. This indicates that fetal infection can take place without high concentrations of BVDV in the uterus or placenta of acutely infected cows and that this is a more likely cause of abortion than replication of the virus in the placenta.

Persistent infections

Abortion is not the only outcome of infection in the first trimester of gestation. Infection of the fetus with an ncp BVDV may result in the birth of a PI animal. Generation of PI animals peaks when infection occurs from about 30 until 90 days of gestation and becomes less frequent as the fetus approaches 125 days of gestation (Roeder et al., 1986; Radostits and Littlejohns, 1988; Moennig and Liess, 1995). Persistent infection has been established with both BVDV 1 and BVDV 2. In PI fetuses, BVDV is widely distributed throughout the organs (Fredriksen et al., 1999b). Damage to the fetus is limited and thus pregnancy is maintained. The fetus is immune-incompetent at the time of infection and mounts no reaction to the infecting BVDV. The continued presence of large amounts of virus throughout fetal tissues tolerizes the fetus, when immune competence sets in. Thus the infecting strain of BVDV is not recognized as foreign and there is no immune response to it.

The effect of the persisting BVDV on the fetus and newborn varies (McClurkin et al., 1979; Liess et al., 1984). Persistent fetal infection may result in the birth of normal calves, apparently normal calves with different functional defects or calves with more overt defects. Most have been characterized as “poor-doers.” They may be stillborn or may die within the first few hours or days of life. Surviving calves are often stunted and have growth retardation. In the first year of life, up to 50% higher death rate was found in PI calves when compared to calves not infected (Duffel and Harkness, 1985). Even apparently normal calves have a higher death rate than uninfected calves, because they are predisposed to infections and have a higher risk of severe illness (Barber et al., 1985; Werdin et al., 1989; Muñoz-Zanzi et al., 2003). This is most likely due to functional defects of the immune system causing im-

mune suppression (Roth et al., 1981, 1986). Persistent infection may also cause functional defects of the endocrine system: Diabetes mellitus has repeatedly been reported in PI animals (Taniyama et al., 1995; Buckner, 1997; Murondoti et al., 1999). It has been suggested that different clinical signs associated with persistent infection are predominantly influenced by the time when the fetus becomes infected with earlier infections, resulting more frequently in the birth of normal, healthy virus carriers (Moennig and Liess, 1995).

Teratogenic effects

Congenital defects have been described after infection between days 80 and 150 of gestation. The fetus appears to be the most susceptible to teratogenic effects of BVDV when the immune competence begins to develop, the ability to mount an inflammatory response sets in, and organogenesis is not completed (Duffel and Harkness, 1985).

Intrauterine growth retardation and reduced maturation are observed in numerous tissues, including brain, thymus, muscles, bone, and lung (Done et al., 1980). Lesions in the brain and eyes are most frequent because they are in the final stages of organogenesis during day 100 and 150 of gestation (Duffel and Harkness, 1985). Alterations affecting both organs are referred to as oculocerebellar syndrome (Bielefeldt Ohmann, 1984). The following malformations of the central nervous system have been reported: micrencephaly, cerebellar hypoplasia or aplasia, porencephaly, hydranencephaly, hydrocephalus internus, or dysmyelogenesis (Ward, 1969; Kahrs et al., 1970 a,b; Casaro et al., 1971; Brown et al., 1973, 1974; Scott et al., 1973; Done et al., 1980; Trautwein et al. 1986). Neurological signs of tremor, ataxia, torticollis, or opisthotonus may be seen in newborn calves (Liess et al., 1984). The ocular abnormalities are characterized by reduced pigmentation of the retina, retinal atrophy, optic neuritis, cataract, microphthalmia, and retinal dysplasia (Kahrs et al., 1970b, Brown et al., 1975). Another frequently affected organ is the thymus, which has reduced size at parturition (Done et al., 1980). Other congenital defects are arthrogryposis and alopecia/hypotrichosis (Casaro et al., 1971; Kendrick, 1971; Bielefeldt-Ohmann, 1984). Frequently BVDV cannot be isolated from or detected in non-PI animals with BVDV-related malformations. In the few cases where BVDV is detected, it is often found locally in the brain or in the cerebrospinal fluid only (Roeder et al., 1986; Trautwein et al., 1987; Liess et al., 1987). Some of the newborns with BVDV-related

malformations are seropositive for BVDV at birth (Liess et al., 1984).

PERSISTENT INFECTIONS

The ability to establish prolonged replication of virus in vivo is important both for the epidemiology and the pathogenesis of BVDV infections. There are two forms of prolonged viral replication. The first form is characterized by viral persistence after intrauterine infection, which results in animals that never clear the virus and thus shed the virus over their entire life span. The second form occurs in some, but not all, animals following acute infection. Such animals shed virus for weeks and months following infection. However, these animals do mount an immune response to the virus and do clear the virus from most tissues over time.

VIRUS PERSISTENCE FOLLOWING INTRAUTERINE EXPOSURE

The more important form of prolonged viral replication results from intrauterine infection of an immune-incompetent fetus because

- PI animals are the main source of virus for establishing acute infections.
- PI mothers are important in establishing PI families by continuous vertical infection.
- Persistent infection is important in the pathogenesis of mucosal disease.

PI animals are characterized by a wide distribution of BVDV throughout their organs, no virus-associated morphological lesions, and no immune response to the persisting BVDV strain. The immune tolerance is selectively restricted to the particular strain of BVDV that has caused the intrauterine infection. Infections with other strains of BVDV will induce an immune response (Fulton et al., 2003). Even single amino acid differences are sufficient for the PI animal to recognize and mount a response to another BVDV strain (Collen et al., 2000). PI animals have normal cellular and humoral responses to other antigens (Houe and Heron, 1993). Recent investigations have centered on immunological mechanisms involved in maintaining viral persistence. It was concluded that tolerance is maintained by nonreactive CD4⁺ T-lymphocytes, and B-lymphocytes and antigen-presenting cells are normally reactive (Fray et al., 2000b; Glew and Howard, 2001).

PI animals as source of virus for acute infection

Some PI calves are born without clinical signs of the disease and without any macroscopic or micro-

scopic lesions (Done et al., 1980; Binkhorst et al., 1983; Roeder et al., 1986). These clinically inconspicuous PI animals are an important source for horizontal and vertical infections. It was demonstrated experimentally that transmission of BVDV to naive animals was much more efficient from PI animals than from those with acute infections (Traven et al., 1991; Niskanen et al., 2000). In PI animals, BVDV is present throughout all organs and tissues (Meyling, 1970; Bielfeldt Ohmann, 1988; Liebler et al., 1991), and thus large scale virus shedding occurs from multiple sites in PI animals. Because BVDV is present in the epidermis; salivary glands; nasal glands; lining of the oral and nasal cavity; and mucosa and accessory glands of the digestive tract, urogenital tract, and mammary glands, the virus can be found in sloughed-off skin cells, oronasal fluids, feces, urine, semen, and colostrum/milk of PI animals. Viral antigen is present in the blood although levels of viremia may change (Brock et al., 1998); thus iatrogenic transmission by contaminated needles or surgical instruments is possible.

Vertical infection

PI cows are able to conceive and give birth to live calves. They have a reduced reproductive performance, which is caused by significant morphological changes in the ovaries leading to reduced follicular maturation (Grooms et al., 1996). Calves born to PI cows are always persistently viremic; this establishes persistent infection in the next generation, thus forming PI families (Straver et al., 1983).

BVDV transmission may occur at the level of oocytes, which can be antigen-positive (Fray et al., 1998). On the other hand, since only about 20% of follicles are positive for BVDV in PI cows (Fray et al., 1998), virus-free calves can be obtained after embryo transfer from PI donors (Wentink et al., 1991; Bak et al., 1992; Smith and Grimmer, 2000). If the oocytes are not infected, infection of the fetus will occur when it becomes susceptible in the early phase of gestation by the continuous presence of BVDV in the uterus, placentomes, and fetal membranes of pregnant PI cows (Fredriksen et al., 1999b). Apparently, pregnancy even enhances the amount of BVDV in the genital tissues (Fredriksen et al., 1999b).

PROLONGED VIRAL SHEDDING FOLLOWING ACUTE INFECTION

A prolonged period of viral shed may develop after acute infection of immune-competent animals. Immune-suppressive treatment or stress during in-

fection may lead to an extended presence of virus in the host for up to 80 days postinoculation (Sandvik et al., 2000). Sometimes virus is retained in certain immunologically privileged sites although a systemic immune response is mounted and virus cleared from most tissues. This type of prolonged viral replication has been described for the male genital system where semen contaminated with BVDV was found for up to 7 months after acute infection (Kirkland et al., 1991; Voges et al., 1998; Niskanen et al., 2002a; Givens et al., 2003).

MUCOSAL DISEASE

Mucosal disease (MD) is the most dramatic clinical evidence of BVDV infection and causes characteristic lesions. The name *mucosal disease* was coined by Ramsey and Chivers (1953). The authors observed a virus infection in cattle that caused severe erosions, ulcerations, and hemorrhages of the mucosal surfaces of nuzzle, oral cavity, esophagus, forestomachs, abomasum, and the small and large intestines. Further examination revealed severe depletion of spleen, lymph nodes, and thymus. These findings are in accordance with later descriptions by other authors (Schulz, 1959; Bielefeldt Ohmann, 1983; Bolin et al., 1985b; Wilhelmssen et al., 1991). The disease is fatal and the affected animals usually die within 2 weeks after onset of clinical symptoms.

PATHOGENESIS OF MUCOSAL DISEASE

Gillespie et al. (1960) recognized that the virus of mucosal disease was identical with one that causes bovine virus diarrhea (Olafson et al., 1946). Inoculation of immune-competent cattle with BVDV-induced acute BVDV infections and not MD. Although acute BVDV infections infrequently resulted in severe disease with mucosal lesions, they tended to be much more limited and less severe than MD. The pathogenesis of MD remained obscure for several decades. It was only when it was recognized that viral persistence after intrauterine infection of the fetus with ncp BVDV was a prerequisite for the development of MD (Coria and McClurkin, 1978; McClurkin et al., 1984) that researchers were able to induce MD. The first two research teams to induce MD experimentally used slightly different approaches:

- One group inoculated pregnant naive cows during the first trimester of pregnancy with the ncp BVDV of an ncp/cp BVDV pair isolated from an animal with MD. This resulted in the birth of PI calves (McClurkin et al., 1984). When these calves were infected with the cp part of the

BVDV pair, they died from MD (Bolin et al., 1985b).

- The other group identified a herd that contained a number of PI animals. One of these animals developed spontaneous MD. A cp BVDV was isolated from the MD animal. When the remaining PI animals were inoculated with the cp BVDV isolate from the diseased animal, they developed the characteristic signs of MD and died within 2–3 weeks (Brownlie et al., 1984).

Several earlier experiments had revealed that not all experimental infections of PI animals with cp BVDV resulted in MD (Liess et al., 1974; Harkness et al., 1984). The comparison of ncp/cp BVDV pairs from animals with MD showed a very close antigenic relationship between ncp and cp of most virus pairs (Howard et al., 1987). Therefore it was hypothesized that only cp BVDV with close antigenic resemblance (homologous strains) can induce MD (Brownlie and Clarke, 1993; Nakajima et al., 1993). In experimental infections with in vitro selected cp BVDV strains, this could not be confirmed, although close homology appears to favor the development of MD as demonstrated in the following experiments:

- Cytopathic BVDV strains with similar, but not completely identical, reactivity patterns to ncp viruses isolated from PI animals were selected. Reactivity patterns were determined based on binding of monoclonal antibodies produced against the immune dominant surface glycoprotein E2. When the PI animals were inoculated with the selected “homologous” cp BVDV, MD was induced in all animals within 2–3 weeks (Moennig et al., 1990).
- PI animals were inoculated with semihomologous cp BVDV strains. Only some animals succumbed to MD (Bruschke et al., 1998b; Loehr et al., 1998)
- PI animals developed MD after inoculation with an antigenically different cp BVDV (Sentsui et al., 2001).

This led to the hypothesis that in addition to the homology between ncp and cp BVDV, other factors influence the outcome of infection (Bruschke et al., 1998b). Under experimental conditions, MD was reproduced by nasal or intravenous inoculation with cp BVDV. Under field conditions, horizontal infection is possible, especially from animals in the final stages of MD, which shed both biotypes of BVDV. Endogenous development of cp BVDV in PI animals has been discussed as an alternative way of in-

fection (Brownlie and Clarke, 1993; Fricke et al., 2001). When homologous cp BVDV has developed in a PI animal, it will develop MD. During the disease, cp BVDV is excreted and other herd members are infected. Seropositive animals are immune, seronegative animals will develop acute BVD, and other PI herd members infected with the same ncp BVDV will develop MD.

EARLY AND LATE ONSET MUCOSAL DISEASE

In initial experiments, the first signs of MD developed within 2–3 weeks after inoculation with cp BVDV. However, later reports described the development of MD several months after inoculation with cp BVDV (Brownlie and Clarke, 1993). It was postulated that the course of infection was influenced by the degree of homology between the persisting ncp BVDV and the cp BVDV (Brownlie and Clarke, 1993). Experimental infections of PI animals using cp BVDV strains of variable degrees of antigenic homology as determined by monoclonal antibody reactivity pattern showed that the course of disease is not predictable (Loehr et al., 1998). The following pattern was observed in animals developing MD months to years after inoculation with cp BVDV. An initial, transient acute BVDV infection with the cp

BVDV was followed by a variably long phase without clinical signs and neutralizing antibodies to the cp BVDV. The final phase of infection was denoted by a sudden onset of characteristic signs of MD (Loehr et al., 1998).

Moennig et al. (1993) compared the cp BVDV used for inoculation and the cp BVDV re-isolated from the sick animal from animals that had succumbed to MD months to years after experimental infection. It was found that the re-isolated cp BVDV was a recombination of the cp BVDV used for inoculation with the persisting ncp BVDV. The E2-specific monoclonal antibody reactivity pattern of the re-isolated cp BVDV resembled that of the persisting ncp BVDV. However, it expressed the genetic marker of the cp BVDV used for inoculation. The recombinational events were confirmed on a molecular basis by sequencing the E2 and NS2/3 proteins of the viruses involved (Fritzemeier et al., 1995, 1998). The same phenomenon has been described when modified live vaccines containing cp BVDV are used (Ridpath and Bolin, 1995). Such recombinational events may even occur between BVDV 1 and BVDV 2 (Ridpath and Bolin, 1995).

Based on these findings *early onset* and *late onset* MD can be distinguished as follows (Figure 7.4): Early onset MD occurs within 2–3 weeks after ex-

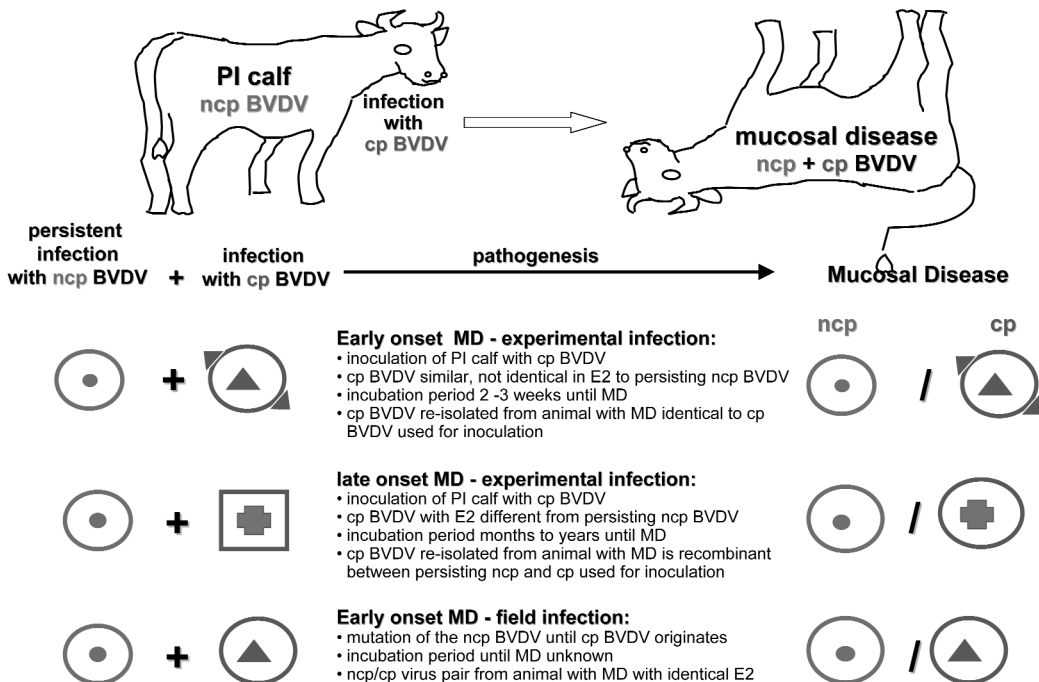


Figure 7.4. Pathogenesis of mucosal disease.

posure to a cp BVDV strain, and the re-isolated cp BVD is identical to the one the animal was exposed to. Late onset MD occurs months to years after exposure and the re-isolated cp BVDV is a recombination between the persisting ncp and the original cp BVDV. This distinction is in general possible only under experimental conditions, because in most field cases the identity of the original infecting cp BVDV cannot be determined and the time of exposure cannot be pinpointed. Clinical signs in the end phase of early onset and late onset MD are indistinguishable and there are only subtle differences in tissue lesions of animals that have succumbed to the two forms of MD (Moennig et al., 1993; Liebler-Tenorio et al., 2000).

PATHOGENESIS OF LESIONS IN MUCOSAL DISEASE

Correlation between viral antigen and tissue lesions

In field cases of MD, BVDV antigen can be demonstrated by immunohistochemistry in numerous organs and tissues (Meyling, 1970; Bielefeldt Ohmann, 1983). The distribution of viral antigen does not correlate well with tissue lesions, since in general no distinction can be made between the antigen of the ncp and cp BVDV. The ncp BVDV present in PI animals in a wide distribution is still found in animals after they have developed MD, but is not associated with tissue lesions (Bielefeldt Ohmann, 1988). Under experimental conditions, it is possible to selectively demonstrate the distribution of cp BVDV as opposed to the persistent ncp BVDV (Liebler et al., 1991). These conditions require that the cp BVDV used for inoculation differs in binding to one or more monoclonal antibodies from the persisting BVDV. Thus it can be recognized selectively by specific monoclonal antibodies in tissue sections using immunohistochemistry. Further studies indicated that the organ distribution patterns for the cp BVDV were identical whether immunohistochemistry for E2 or whether molecular detection of NS3 protein characteristic for the cp BVDV was used (Greiser-Wilke et al., 1993). This suggests that results obtained by immunohistochemistry reflect the distribution of cp BVDV in the tissues realistically.

Examination of animals with MD revealed that cp BVDV was predominantly detected in sites where tissue destruction was observed (Liebler et al., 1991). The sites included

- Depleted lymphoid follicles of tonsils, lymph nodes, spleen, mucosa-associated lymphoid

tissues (gut-associated lymphoid tissues, bronchus-associated lymphoid tissues)

- Thymic cortex
- Altered mucosa of the upper digestive tract, and (iv) crypt epithelia of small and large intestine

These observations suggest that cp BVDV is not only important for the pathogenesis of MD, but also for the manifestation of MD at mucosal surfaces and in lymphoid tissues. It appears that the replication of cp BVDV is associated with marked lesions. The comparison of tissue lesions and distribution of viral antigen at consecutive times after inoculation of PI cattle with cp BVDV revealed a spreading pattern for the cp BVDV in MD that was similar to the one described for acute BVDV infections either with cp or ncp BVDV (Liebler-Tenorio et al., 1997) (Figure 7.5).

After intranasal inoculation, primary infection of the tonsillar epithelium and virus replication in the tonsil is observed. The cp BVDV then spreads from the tonsils to the regional lymph nodes. With little delay, cp BVDV can also be detected in Peyer's patches, mucosa-associated lymphoid follicles in the large intestine, and lymphoid follicles of the lung and nasal mucosa. In mucosa-associated lymphoid tissues, the cp viral antigen is primarily found in the lymphoid tissue and only later in the epithelium. This pattern differs from most enteric pathogens. It suggests a hematogeneous spread of cp BVDV to the gastrointestinal and respiratory tract mucosa. cp BVDV spreads to a lesser degree to the peripheral lymph nodes, thymus, and spleen.

Infection of intestinal mucosa occurs initially in close association with mucosa-associated lymphoid tissues by local expansion of the infection. In the progressed phase of MD, cp BVDV is found multifocally and finally diffusely in the intestinal mucosa. The multifocal infection is most likely caused by multifocal immigration of infected lymphocytes into the mucosa. A large number of these cells might be recirculating from Peyer's patches, since homing is a known phenomenon of the Peyer's patch lymphocytes. The preferential infection of the intestinal mucosa versus other mucosal surface is most likely influenced by the large amount of mucosa-associated lymphoid tissue in the intestine and the high numbers of recirculating lymphocytes. When cp BVDV is present multifocally or diffusely in the intestinal mucosa, the first signs of diarrhea are observed.

Lesions in lymphoid tissues

In the early phase of MD the increased frequency of cell death by apoptosis and the changes in prolifer-

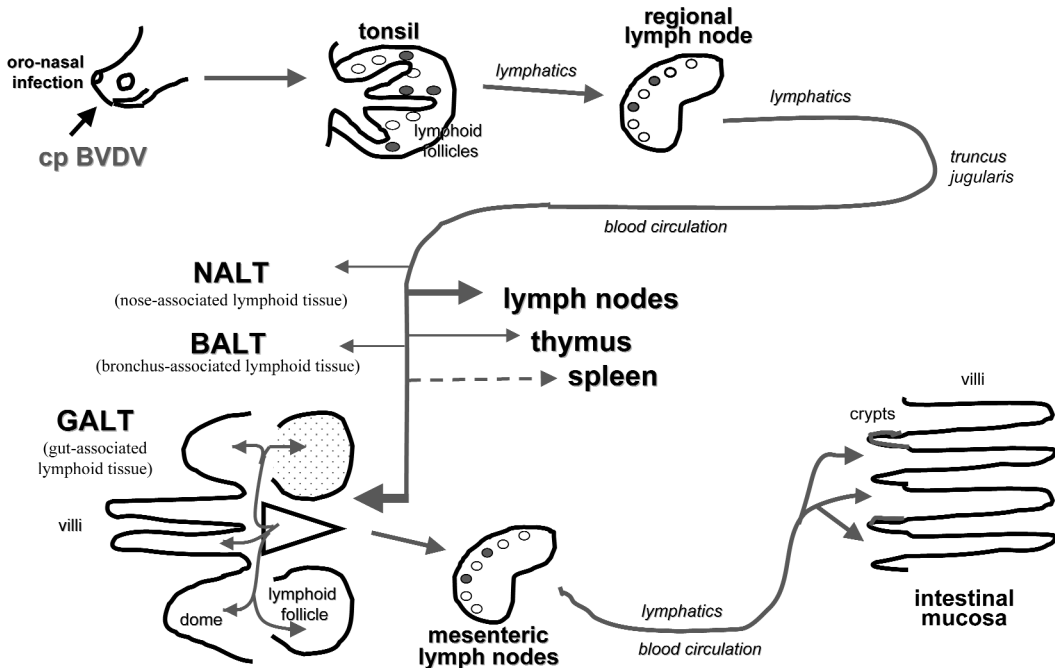


Figure 7.5. Spread of cp BVDV in mucosal disease.

ation are key players for the development of tissue lesions both in lymphoid tissues and epithelium. In tonsils, lymph nodes and Peyer's patches, cp BVDV is predominantly present in the lymphoid follicles. Initially it is associated with follicular dendritic cells (FDCs) and B-lymphocytes. There is a marked reduction in the number of proliferating B-lymphocytes and a marked increase in apoptotic cells. The immature B-lymphocytes in the dark zone of the germinal center, which have a very high proliferative activity, are especially affected (Liebler-Tenorio and Pohlenz, 1997). Transmission electron microscopy reveals a loss of B-lymphocytes, whereas FDCs often do not have signs of degeneration (Teichmann et al., 2000). If the loss of B-lymphocytes is very abrupt, death of FDCs is observed leaving empty follicular centers. Histologically this presents as cyst formation, a frequent finding in mucosa-associated lymphoid tissues of the intestine, which has led to the descriptive diagnosis of "colitis cystica." If depletion of lymphocytes develops more slowly, follicles consisting exclusively of FDCs are found.

Cp BVDV was also detected in dendritic cells (DCs) in the interfollicular areas. DCs can be infected with virus and may serve as carriers for viral proteins (Sprecher and Becker, 1993). Since DCs are able to present antigen very effectively to

T-lymphocytes, they may induce a cell-mediated immune response. Further investigation of T-cell subsets in the early phase of MD revealed accumulations of CD4+ T-lymphocytes particularly at sites where lesions develop and it was speculated about possible cytotoxic effects mediated by CD4+ T-lymphocytes (Frink et al., 2002).

Lesions in mucosa-associated lymphoid tissues

Besides changes within the lymphoid follicles, the loss of B-cells also alters the morphology of domes (Liebler et al., 1995). The loss of domes, and consequently of the follicle-associated epithelium, impedes the selective uptake of antigens from the intestinal lumen. Thus MD causes severe impairment of the inductive part of the intestinal mucosal immune system.

The changes in the gut-associated lymphoid tissue cause not only local tissue destruction, but also affect the whole intestinal mucosa (Liebler et al., 1996). In the mucosa of the small and large intestines of animals moribund with MD, the number of IgA- and IgM-positive plasma cells in the lamina propria is severely reduced. This is most likely due to the depletion of mucosa-associated lymphoid follicles, which are the sites where precursors of the plasma cells in the lamina propria are generated. The reduced number of plasma cells causes reduced

production of secretory immunoglobulins essential for the protection of the mucosal surface.

The number of intraepithelial lymphocytes is also reduced (Liebler et al., 1996). Since they are also derived from Peyer's patches in part, their reduced numbers might reflect decreased production. On the other hand, the altered microenvironment of the epithelium might also influence their presence between the epithelial cells. In conclusion, all parts of the mucosal immune system are severely altered in MD—e.g., FAE, domes, lymphoid follicles, plasma cells in the lamina propria, and T-lymphocytes in the epithelium.

Lesions in mucosa

The intestinal mucosa becomes infected later than the Peyer's patches and mucosa-associated lymphoid follicles in the large intestine (Liebler-Tenorio et al., 1997). Enterocytes are not infected from the luminal surface as in most enteric viral infections, but from the basolateral surface. Crypt epithelium is particularly affected. Initially there is a coincidence of three phenomena in the crypts; focal infection of a few epithelial cells with cp BVDV, increased number of apoptotic epithelial cells, and increase of epithelial proliferation rate (Liebler-Tenorio and Pohlenz, 1997). The induction of apoptosis in the crypt epithelium appears to play a key role in the destruction of the intestinal mucosa. In affected crypts, the proliferation rate is severely reduced, and there is a reactive increase of proliferating cells in adjacent crypts.

A common finding in moribund animals in the late phase of MD is the multifocal to diffuse infection of the intestinal mucosa with cp BVDV. In the mucosa of these animals, an increasing number of crypts is without proliferating epithelial cells. As soon as the continuous cellular loss of aged cells from the villous tips is not compensated by crypt proliferation, mucosal atrophy and ulceration develop. At this stage of infection, clinical symptoms of diarrhea become apparent. The progressive lesions explain the lack of success in treating apparently sick animals.

CONCLUSIONS

Many pieces of the complex puzzle of BVDV pathogenesis have been put in place over the last decades, but important pieces are still missing. Observations from experimental infections and naturally occurring disease continue to create doubt about some of the concepts of pathogenesis. Currently, advances are being made in elucidating the interaction of BVDV

infection with different components of the specific immune responses (Beer et al., 1997; Rhodes et al., 1999; Collen and Morrison, 2000; Collen et al., 2002), as well as with cells and mediators of the innate immune system (Adler et al., 1994, 1996; Baigent et al., 2002; Ganheim et al., 2003; Peterhans et al., 2003). Another approach focuses on the interaction of BVDV with its target cells—e.g., how BVDV replication influences gene expression in affected cells (Neill and Ridpath, 2003), in which compartments of the cell BVDV proteins are expressed (Grummer et al., 2001); which receptors are used by BVDV to interact with cells (Minocha et al., 1997; Schelp et al., 2000); and how cp strains are able to exert their cp effect on certain, but not all, cells (Perler et al., 2000; Schweizer and Peterhans, 1999, 2001; Grummer et al., 2002; Bendfeldt et al., 2003). Hopefully some of the answers will yield further insight into the pathogenesis of BVDV and allow better control of this economically important disease of ruminants.

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8

Reproductive Disease and Persistent Infections

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INTRODUCTION AND OVERVIEW

Bovine viral diarrhea virus (BVDV) is recognized as one of the most important infectious diseases of cattle (Baker, 1987; Duffell and Harkness, 1985). The insidious nature of BVDV contributes to substantial economic losses in both the dairy and the beef cattle industries on a worldwide basis (Duffell et al., 1986; Houe et al., 1993a). The most common consequences of BVDV infection in cattle are respiratory and reproductive disorders. Losses due to BVDV-related reproductive disorders are probably the most economically important consequence, and there is evidence to suggest that such losses are increasing in the United States (Evermann and Ridpath, 2002). Although difficult to measure, the economic consequences of reproductive infections can be devastating due to negative effects on the individual animal and on the net return per cow. In addition to reduced reproductive efficiency, BVDV has adapted to the bovine reproductive system, thereby maintaining itself in the cattle population by inducing immunotolerant fetal infections; this results in the birth of calves persistently infected with BVDV. The primary consequences of reproductive infection are due to the direct infection of the fetus. In turn, persistently infected (PI) animals act as a source of BVDV within the cattle populations.

Reproductive losses associated with BVDV infection were initially described by Olafson and associates in the first clinical description of bovine viral diarrhea (Olafson et al., 1946). Pregnant cows that were subclinically infected with BVDV, often aborted 10–90 days following infection. It has been observed that BVDV can cause a wide array of reproductive losses manifested in many different clin-

ical pictures. The outcome of BVDV infection depends primarily on the stage of the reproductive cycle or gestation.

IMPACT OF TESTICULAR INFECTION ON BULL FERTILITY

The influence of BVDV infection on bull fertility and reproduction is often overlooked. Both acute and persistent infections can affect the reproductive soundness of bulls by reducing semen quality (Coria and McClurkin, 1978; Barlow et al., 1986; Meyling and Jensen, 1988; Revell et al., 1988; Kirkland et al., 1994; Whitmore and Archbald, 1977; Kirkland et al., 1991; Kommisrud et al., 1996). The primary effect of infection is the subsequent potential for venereal transmission during breeding and the shedding of BVDV in semen. Virus has been isolated from the semen of PI bulls ranging from 10^4 to 10^7 CCID₅₀/ml of semen (Kirkland et al., 1991). Following acute experimental infection of bulls with noncytopathic BVDV, virus was isolated from the semen between 7 and 14 days postinfection at titers ranging from 5–75 CCID₅₀/ml of semen (Kirkland et al., 1991). The ability to transmit BVDV via semen is supported by the demonstration that susceptible cows can become infected following artificial insemination (Kirkland et al., 1994; Paton et al., 1990) or natural service (McClurkin et al., 1979; Wentink et al., 1989).

Persistently infected bulls can successfully sire offspring but usually with lowered conception rates (Meyling and Jensen, 1988; Kirkland et al., 1994; Paton et al., 1990). Poor reproductive efficiency following the use of PI bulls is likely attributable to a combination of several factors including low quality semen, ill thrift, and the effects of the virus on the re-

productive tract and conceptus of infected females. The quality of semen from PI bulls may range from acceptable (Barlow et al., 1986; Kirkland et al., 1991) to abnormal with various defects predominantly involving the head of the spermatozoa (Barlow et al., 1986; Revell et al., 1988; McClurkin et al., 1979). Prior to the development of standardized BVDV testing protocols in semen collection/production facilities, semen was unknowingly collected and distributed from PI bulls. A retrospective analysis of breeding records in 97 dairy farms in which semen from a PI bull was used, indicated a first service conception rate of 38% in cows inseminated with this semen as compared to 66% for cows bred during the same period on the same farms with semen from a different bull (Kirkland et al., 1994).

Following acute infection, bulls can shed BVDV in semen (5–75 CCID₅₀/ml of semen). In contrast to semen from PI bulls, the semen of acutely infected animals (based on the criteria of concentration, motility, and morphology of spermatozoa) will pass breeding soundness examination (Kirkland et al., 1997; Kirkland et al., 1991). It is generally accepted that the ability to isolate virus from semen ceases when serum antibodies become detectable (Kirkland et al., 1991; Paton et al., 1989). BVDV isolation from raw semen may be less successful than from extended semen (Revell et al., 1988). This is presumably due to the documented viricidal effects of semen. Using semen that was collected prior to seroconversion (12 days postinoculation), approximately 5% of inseminated heifers became infected as evidenced by seroconversion (Kirkland et al., 1997). It is interesting to note that in this report the primary impact of BVDV was the horizontal transmission of BVDV to penmates and the subsequent birth of PI calves (Kirkland et al., 1997).

As is true for most biological systems, there are always exceptions to the rule. In addition to classical acute and persistent infections, the replication of BVDV in a “privileged” site has been described. Thus, persistent BVDV infection localized in the testes of an immunocompetent, seropositive, non-viremic bull has been documented (Voges et al., 1998). The concentration of BVDV in the semen of this bull was lower ($<2 \times 10^3$ CCID₅₀/ml) than that observed in PI bulls (10^4 – 10^7 CCID₅₀/ml), but higher than that observed in acutely infected bulls (5–75 CCID₅₀/ml) (Kirkland et al., 1991; Voges et al., 1998). The insemination of seronegative heifers with semen collected from this bull resulted in BVDV infection and subsequent seroconversion

(Niskanen et al., 2002). In addition, during the reported period of collection, the bull continued to have consistent, high levels of BVDV neutralizing antibody against the viral strain isolated from the semen. Bovine viral diarrhea virus could not be isolated from white blood cells but was continually isolated from semen samples. At postmortem, BVDV was isolated only from the testicular tissue. It is hypothesized that this bull was acutely infected with BVDV near the time of puberty when the blood-testes barrier forms, thus trapping the virus in gonadal cells away from the animal's immune response. These findings suggest that screening bulls for persistent infection with BVDV using serum or white blood cells may not be adequate in assuring BVDV-free semen.

Persistent, localized testicular BVDV infections in experimentally infected, post-pubertal, non-viremic bulls have been characterized (Givens et al., 2003a). Following experimental infection, BVDV persisted within the testicular tissues of some bulls for at least 7 months (Givens et al., 2003a). Experimental results have indicated that an epididymal infection may progress to a testicular infection. Due to the blood-testes barrier, BVDV is protected from elimination by the immune system and testicular infection can persist (Paton et al., 1989; Kirkland et al., 1991). The prevalence of bulls that are non-viremic (based on failure to isolate virus from serum or buffy coat sample) but shed BVDV in semen is probably extremely low (Givens et al., 2003b; Niskanen et al., 2002). Further studies are required to determine whether persistent-testicular BVDV infections contribute to transmission of BVDV by semen to susceptible cows.

Currently, BVDV contamination of distributed semen is prevented by practicing standardized testing and quarantine procedures in AI semen collection facilities. Certified Semen Services, Inc. (CSS) is a cooperative organization, and membership in CSS ensures that the standardized procedures are used appropriately (CSS guidelines: http://www.naab-css.org/about_css/disease_control-2002.html). Therefore, use of semen from CSS-certified collections is recommended to prevent introduction of BVDV via semen. Current CSS prevention measures include screening of all bulls 30 days prior to entry by virus isolation or ELISA to detect persistent infections. The collection and distribution of semen from bulls with persistent testicular infections is prevented by requiring that specimens of semen be negative by virus isolation.

IMPACT OF OVARIAN INFECTION ON REPRODUCTIVE CAPACITY

BVDV infections prior to breeding often go unnoticed in production operations, and thus field data to gauge its impact are sparse. Limited information is available on ovarian function following BVDV infection. Reproductive capacity of bulls and cows/heifers can be affected directly by BVDV infection. Bovine viral diarrhea virus has been identified by virus isolation and immunohistochemistry in ovarian tissues and oviductal cells following acute infection. The identification of BVDV and viral antigen has been associated with chronic oophoritis and salpingitis of cattle. Clinical examination of cattle experimentally infected with BVDV revealed reduced number of corpora lutea and recovered embryos. Between 6 and 60 days following experimental acute infection, viral antigen (E^{ns}) was detected by immunohistochemistry in interstitial stromal cells and macrophage-like cells that were associated with primary follicles, secondary follicles, antral follicles, corpus luteum, and corpus albicans (Grooms et al., 1998a). Viral antigen (E^{ns}) was detected by immunohistochemical techniques in ovarian sections taken on days 10, 20, and 30 after immunization with a modified-live BVDV vaccine (Grooms et al., 1998b). Experimental acute BVDV infection near estrus has resulted in reduced follicular development following infection (Grooms et al., 1998c).

In a study of cattle being superovulated while undergoing experimental challenge with BVDV, the number of palpable corpora lutea and recovered embryos was significantly reduced when compared to noninfected cows undergoing superovulation (Kafi et al., 1994). BVDV infection resulting in viremia during the preovulatory phase can reduce the rate of follicle growth (Grooms et al., 1998a; Fray et al., 1999). Cows that are persistently infected with BVDV have ovaries that are often hypoplastic with a significantly reduced number of ovarian antral follicles when compared to ovaries from cattle not persistently infected with BVDV (Grooms et al., 1996). Alteration of ovarian hormone secretions has been demonstrated following acute BVDV infection and has been postulated to contribute to BVDV-induced infertility (Fray et al., 1999; Fray et al., 2000a; 2000b; 2002). The changes in follicular development and ovarian hormonal dynamics associated with BVDV infection may subsequently lead to a transient and/or long-term reduction in fertility.

IMPACT OF STAGE OF GESTATION ON THE OUTCOME OF REPRODUCTIVE DISEASE

As stated previously, the outcome of reproductive disease depends on the stage of gestation at which fetal infection occurs. Abortions associated with BVDV infection were first described in 1946 (Olafson et al., 1946). Initial reports linked abortions to epizootics of disease described as bovine viral diarrhea although definitive causes of abortions were not identified (Dow et al., 1956; Nielson et al., 1955; Swope and Luedke, 1956). Early studies involving experimental infection with BVDV resulted in abortion although virus was not isolated from the fetus (Baker et al., 1954; Huck, 1957). Subsequently noncytopathic (Gillespie et al., 1967) and cytopathic BVDV (Shope, 1968; Scott et al., 1972) were isolated from aborted fetuses. Experimental, transplacental infection of fetus with BVDV was first demonstrated in 1969 (Ward et al., 1969). Under experimental conditions, both cytopathic (Brownlie et al., 1989) and noncytopathic BVDV (Done et al., 1980; Duffell et al., 1984; Liess et al., 1984) can cause fetal death following infection of seronegative dams. An abortion rate of 21% in a 6-month period has been documented to have occurred in a susceptible herd following the introduction of BVDV in the herd (Roeder et al., 1986). In endemically infected herds without BVDV prevention and control programs (vaccination, biosecurity, test, and removal), it has been estimated that 7% of fetal deaths may be attributable to infection with BVDV (Rufenacht et al., 2001).

Fetal death following BVDV infection of susceptible dams can occur at any stage during gestation, although abortions are most common during the first trimester (Duffell and Harkness, 1985; Done et al., 1980; Roeder et al., 1986; Casaro et al., 1971; Kahrs, 1968; Kendrick, 1971; Sprecher et al., 1991; McGowan et al., 1993). However, BVDV can also be associated with late-term abortions. In a field investigation of an abortion outbreak in a large dairy operation, BVDV was isolated from 18 fetuses, 14 of which were aborted during the last trimester of gestation (Grooms, unpublished data). Depending on the age of the fetus, fetal reabsorption, mummification, or expulsion can occur following infection with BVDV (Done et al., 1980; Casaro et al., 1971).

CONSEQUENCES OF BVDV INFECTION PRIOR TO IMPLANTATION (30–45 DAYS)

Understanding the effects of BVDV infection during the early stages of embryo development has proven

to be difficult. Characterization of the complex events that influence conception is difficult and contributes to the lack of understanding of the effects of BVDV. From epidemiological and clinical observations it is apparent that BVDV infection reduces not only the conception rates but also the viability of the early stage conceptus. Conception rates were reduced in cattle exposed to BVDV during breeding when compared to cattle seropositive to BVDV (Houe et al., 1993a, 1993b; McGowan et al., 1993; McGowan et al. 1995; Virakul et al., 1988). The mechanism(s) for reduced conception is not directly known. However, it has been demonstrated that BVDV infection results in the replication of BVDV in ovarian tissues (Grooms et al., 1998a, 1998b). The most common consequence of BVDV infection during the early stages of gestation is infertility. In vitro experimental studies have demonstrated that the zona pellucida provides a protective effect to the early developing embryo.

When a group of seronegative cattle was accidentally exposed to a persistently infected cow during breeding, the conception rates in cattle that seroconverted to BVDV before, during, or after breeding were 78.6%, 44.4%, and 22.2%, respectively (Virakul et al., 1988). Cattle that seroconverted to BVDV at breeding or soon after breeding were less likely to conceive than cattle that had seroconverted prior to breeding (Virakul et al., 1988). McGowan (1993) compared BVDV seropositive heifers to heifers that seroconverted between breeding and pregnancy diagnosis at 51 days postinsemination and found that the pregnancy rate was significantly lower in heifers seroconverting following breeding. Houe et al. (1993b) identified and defined a specific risk period for BVDV infection in dairy herds in which cattle persistently infected with BVDV were present. The risk period was defined as the period of time previous to when the oldest PI animal was 6 months old. In all herds studied, conception rates were significantly lower during the defined risk period than during the post-risk period (Houe et al., 1993b). In an experimental study examining BVDV infection around breeding, conception rates in heifers infected intranasally 9 days before insemination was 44% compared to 79% for the control group (McGowan et al., 1993). The reduction in conception rates was attributed to either failure of fertilization or early embryonic death. In the same report, the conception rate in heifers exposed to a persistently infected cow and calf 4 days following insemination was 60% (McGowan et al., 1993). However, significant embryo loss was experienced

in this group, resulting in a 77-day pregnancy rate of only 33% as compared to 79% for the control group.

The mechanism for decreased conception rates is not clear but may depend on the time of infection with respect to the stage of early reproductive events. Virus has been localized in ovarian tissue for prolonged periods of time following acute infection with cytopathic (Ssentongo et al., 1980; Grooms et al., 1998b) and noncytopathic BVDV (Grooms et al., 1998a). BVDV has also been isolated from follicular fluid collected from slaughterhouse ovaries (Bielanski et al., 1993). Exposure of developing oocytes to BVDV could result in reduced survivability either through direct cell damage or indirectly through changes in the oocyte at the cellular level. Following acute infection with cytopathic BVDV, interstitial oophoritis has been described with lesions lasting up to 60 days (Ssentongo et al., 1980; Grooms et al., 1998a). Significant long-term oophoritis could result in ovarian malfunction with subsequent poor conception rates.

Because of its essential role in fertilization, changes in the oviductal environment could have a detrimental effect on the conception rate. BVDV has been detected in oviductal cells (Bielanski et al., 1993; Booth et al., 1995). Archbald et al. (1973) isolated BVDV from oviductal tissue and detected evidence of salpingitis for up to 21 days following experimental intrauterine infusion with cytopathic BVDV. Similar findings have not been reported with noncytopathic BVDV.

Studies have suggested that the interruption of normal fertilization or embryonic death may be the mechanism for a reduction in conception rates associated with acute BVDV infection. This conclusion was drawn from the observation that infusion of cytopathic BVDV into the uterus at insemination of superovulated cows resulted in a significant reduction in the number of fertilized ova found at slaughter 3 and 13 days later (Grahn et al., 1984). Archbald et al. (1979) provided evidence that BVDV may interfere with early embryonic development. In superovulated cattle in which BVDV had been infused into one uterine horn, the quality of the embryos collected from the infected horn was significantly reduced compared to those collected from the noninfected horn (Archbald et al., 1979). In a similar study, the conception rate in seronegative heifers infused with BVDV 2 hours following breeding was 27% and was significantly reduced (67%) as compared to sham-inoculated cows (Whitmore et al., 1981). However, in the same study, conception rates

of seropositive cows inoculated in utero with BVDV or seronegative cows inoculated orally and intranasally were not significantly different than those in control cows.

Although it is thought that BVDV has a direct effect on the developing embryo, inflammatory changes in the uterus following BVDV infection may result in an incompatible environment for embryo development. In a study of uterine pathology following intrauterine infusion of cytopathic BVDV, histological changes in both the uterus and oviduct were evident from 6–21 days postinfection (Archbald et al., 1973). Several in vitro studies have been undertaken to elucidate the effects of BVDV on early reproductive function. Ova exposed to BVDV in vitro can have virus particles attached to the zona pellucida (Gillespie et al., 1990). However, in vitro studies have shown that the intact zona pellucida protects the developing embryonic cells from BVDV infection, allowing normal development to continue (Singh et al., 1982; Potter et al., 1984). In morula and blastocyst stage bovine embryos with the zona pellucida intact or damaged, no cytopathic effects were seen for 48 hours following exposure to cytopathic BVDV (Bielanski and Hare, 1988). Similarly, zona pellucida intact embryos exposed to noncytopathic BVDV infected bovine oviductal epithelial cells for 7 days showed no adverse effects in their rates of development (Zurovac et al., 1994). In contrast, blastocysts hatched from the zona pellucida (day 8 of gestation) have been shown to have decreased viability when exposed to cytopathic BVDV in vitro. In the same study, noncytopathic BVDV did not decrease blastocyst survivability (Brock and Stringfellow, 1993). These studies suggest that the zona pellucida protects the developing embryo from direct effects of BVDV. However, following removal of the zona pellucida, cytopathic BVDV may have detrimental effects on the survivability of blastocysts. Noncytopathic BVDV has not been shown to have the same effects. Because noncytopathic BVDV is the most common virus isolated in acute outbreaks of BVDV, and has been the biotype associated with reported decreases in conception rates, further characterization of the effect of noncytopathic BVDV on the early stages of the developing embryo is necessary. Interestingly, at day 14 post-hatching, BVDV antigen has been detected in embryos inoculated with noncytopathic BVDV at hatching (Brock and Stringfellow, 1993). In contrast to cytopathic BVDV, it is possible that the effect of exposure of embryos to noncytopathic BVDV may be delayed.

CONSEQUENCES OF BVDV INFECTION FROM 30–125 DAYS OF GESTATION

Following implantation, transplacental infection of the developing fetus can occur in susceptible cows with either noncytopathic or cytopathic strains of BVDV. The outcome of infection is largely dependent on the timing of infection, the immunocompetence of the developing fetus, the virus biotype involved and the virulence of the virus. Although the mechanism of fetal infection is not clear, evidence suggests that BVDV may cross the placenta by causing vasculitis on the maternal side of placentation allowing access to fetal circulation (Fredriksen et al., 1999). Following infection of pregnant animals, BVDV crosses the placenta, and replication can be demonstrated in the fetus within 7 days postinfection.

The phenomenon of persistent infection was first described in an apparently healthy bull (Coria and McClurkin, 1978). Experimental production of persistent infections was reported in 1984 (Brownlie et al., 1984; Liess et al., 1984; McClurkin et al., 1984). During gestation, fetuses from 18–125 days of age that survive noncytopathic BVDV infection develop immunotolerance between 125–150 days of gestation and subsequently harbor lifelong persistent infection. Although there are several hypotheses regarding the mechanism of induction of immunological tolerance, it is clear that the circulation of BVDV during the period of gestation when immunocompetence is developing (90–120 days) is a prerequisite for immunotolerance and persistent infection. Persistent BVDV infection in cattle appears to arise from specific B- and T-lymphocyte immunotolerance (Coria and McClurkin, 1978; McClurkin et al., 1984). In persistently infected animals, BVD viral proteins are accepted as self-antigens with a resulting negative selection or down-regulation of BVDV specific B- and T-lymphocytes during ontogeny. This negative selection results in an absence of neutralizing and non-neutralizing antibodies and of cell-mediated immunity to the persistent virus (Donis and Dubovi, 1987). It appears that the window of opportunity during fetal development for BVDV to establish an immunotolerant infection is approximately 100 days. Under experimental conditions, persistent infection occurred in 86% and 100% of calves derived from cows infected with BVDV at days 18 and 30 of gestation, respectively (Kirkland et al., 1993).

Persistent infections have been obtained in 100% of fetuses in dams challenged at 75 days of gestation (Cortese et al., 1998; Brock and Cortese, 2001;

Brock and Chase, 2000). Although persistent infections have been reported to occur following infection up to 125 days of gestation, they are only rarely established in fetuses older than 100 days of gestation (Baker, 1995). Noncytopathic BVDV is the only biotype that has been observed clinically or experimentally to produce persistent BVDV infections (Brownlie et al., 1984; Brownlie et al., 1989; Bolin et al., 1985). Experimental infection of pregnant dams in the first trimester of gestation with cytopathic BVDV failed to produce persistently infected calves (Casaro et al., 1971; Brownlie et al., 1989; McClurkin et al., 1984). The simultaneous experimental infection of type 1 and type 2 genotypes during gestation has resulted in dual persistent infections with the two BVDV genotypes (Brock and Chase, 2000).

CONSEQUENCES OF BVDV INFECTION FROM 125–175 DAYS OF GESTATION

Fetal infection during fetal development and organogenesis in the middle trimester can result in numerous types of congenital anomalies. The combination of direct cellular damage by virus and the resultant inflammatory response to the foreign viral antigens have been proposed as pathogenic mechanisms for congenital anomalies (Castrucci et al., 1990). Congenital defects of the central nervous system are the most common and may include cerebellar hypoplasia (Kahrs et al., 1970; Scott et al., 1973), microencephaly, hydrocephalus, hydranencephaly (Badman et al., 1981), porencephaly (Hewicker-Trautwein and Trautwein, 1994), and hypomyelination (Binkhorst et al., 1983). Cerebellar hypoplasia is the most commonly recognized congenital defect associated with BVDV infection (Ward et al., 1969; Kahrs et al., 1970; Scott et al., 1973; Wilson et al., 1983). Congenital infection has been correlated with ataxia, tremors, wide-based stance, stumbling without compensation or resolution, “dummy calves,” and failure or inability to nurse. The defects frequently are severe enough that compensation does not occur and the calves may either die or require euthanasia (Baker, 1987). Histologically, the cerebellar lesions have been described as containing reduced numbers of molecular layer cells and granular layer cells (Done et al., 1980; Brown et al., 1973; Brown et al., 1974; Bielefeldt-Ohmann, 1984) and reduced numbers and displaced Purkinje cells (Brown et al., 1973). Fetal cerebellar effects have been seen following infection as early as 79 days and as late as 150 days of gestation (Brown et al., 1973). The severity of cerebellar lesions apparently

increases with the age of the fetus at the time of infection up to 150 days of gestation (Brown et al., 1973). Numerous other congenital defects have been associated with BVDV infection and include hyena disease (Espinhasse et al., 1986) (osteochondrosis), growth retardation, optic neuritis (Bielefeldt-Ohmann, 1984), retinal degeneration (Scott et al., 1973), thymic hypoplasia (Done et al., 1980), hypotrichosis/alopecia (Baker, 1987; Kendrick, 1971), curly haircoat (Larsson et al., 1991), deranged osteogenesis (Constable et al., 1993), microphthalmia (Kahrs et al., 1970; Scott et al., 1973; Brown et al., 1975), cataracts (Bielefeldt-Ohmann, 1984; Wohrmann et al., 1992), mandibular brachygnathism (Scott et al., 1972), and growth retardation (Baker, 1987; Done et al., 1980, Constable et al., 1993). From a diagnostic aspect, it is important to note that it is generally difficult to isolate BVDV or demonstrate viral antigen in calves that exhibit signs associated with the congenital defects as described.

CONSEQUENCES OF BVDV INFECTION FROM 175 DAYS OF GESTATION TO TERM

In the later stages of gestation, immunocompetence, and organogenesis are generally complete. Although abortions and the birth of weak calves have been attributed to infection with BVDV late in gestation (Ward et al., 1969), fetuses infected during this time period are normally able to mount an effective immune response and clear the virus. These calves are usually normal at birth and have precolostral-neutralizing antibodies to BVDV (Casaro et al., 1971; Kendrick, 1971; Braun et al., 1973; Ohmann et al., 1982; Orban et al., 1983). However, calves congenitally infected with BVDV may be at more risk of experiencing a serious postnatal health event. It has recently been described that congenital infections during the last trimester of gestation may negatively affect neonatal performance and survivability (Muñoz-Zanzi et al., 2003). It was reported that calves born with BVDV-neutralizing antibodies were twice as likely to experience a severe illness during their first 10 months of life as compared to calves born free of neutralizing antibodies. Further studies are needed to determine whether detrimental effects may continue long-term (Muñoz-Zanzi et al., 2003).

IMPACT OF PERSISTENT INFECTION AND THE RELATIONSHIP TO BVDV-ASSOCIATED DISEASE

As discussed previously, there are several outcomes of BVDV infection during gestation. However, fetal

infection resulting in persistent infection has severe impact on BVDV-associated losses. It is universally accepted that persistent infections are the primary method for the spread of BVDV and the maintenance of the virus in the cattle population. The birth of PI animals leads to increased calf mortality and respiratory tract disease. The PI animal is most commonly the source of BVDV spread when different groups of cattle are mixed. The birth of PI calves commonly occurs following the purchase of pregnant animals. Therefore, the ability of BVDV to cause persistent fetal infection has a significant impact on the continued ability of BVDV to cause disease. Persistent infections occur in spite of using vaccines that effectively modulate and prevent acute disease.

IMPACT OF VIRAL FACTORS ON THE OUTCOME OF REPRODUCTIVE DISEASE

The genotypic classification (1a, 1b, and 2) of BVDV strains is based primarily on antigenic differences in the E2 glycoprotein and comparative nucleotide sequencing. All BVDV genotypes (1a, 1b, and 2) are capable of causing persistent infections. In addition, persistent infection can be caused simultaneously by a mixture of type 1 and type 2 BVDV (Brock and Chase, 2000). Certain BVDV outbreaks involving type 2 genotypes have been associated with increased rates of abortion, which would result in a reduced number of PI calves being born following an outbreak. It has been demonstrated that the distribution of type 1 and type 2 persistent infections in field studies is approximately equal (Wittum et al., 2001).

Differences between cytopathic and noncytopathic BVDV affect the outcome of reproductive infections. Persistent infection is the result only of noncytopathic BVDV infection. Cytopathic BVDV replicates in the reproductive tract, and vaccine origin virus has been isolated in ovarian tissues following vaccination. Transplacental transmission of cytopathic BVDV to the fetus is rare as opposed to rapid transplacental transmission of noncytopathic strains. The mechanism for transplacental transmission of BVDV is unknown but is thought to be related to viremia in the pregnant dam. The mechanism influencing the differential ability of noncytopathic and cytopathic BVDV to infect the fetus is unknown. It is unclear why cytopathic BVDV does not establish persistent infection in the fetus.

Although several published studies are available on the mechanisms of persistence and the role of interferon (IFN) during pregnancy, their findings are

conflicting. Infection of the fetus with cytopathic BVDV leads to IFN production in the fetus that may prevent the establishment of persistent infection. In experimental studies, ncp BVDV fetal infection did not induce IFN- α production. However, ncp BVDV fetal infection did result in IFN- α production in the cow (Charleston et al., 2001). It has been hypothesized that the protective occurrence of apoptosis and the IFN response seen during the earliest stages of pregnancy are important elements of the innate immune system protecting the fetus. Evasion of these key elements may be crucial not only for transmission to the fetus, but also for the maintenance of immunotolerance (Charleston et al., 2002).

Experimental data indicate that immunosuppression caused by ncp BVDV infection may not be associated with low interferon responses or elevated levels of TGF- β (Schweizer and Peterhans, 2001). However, it does not exclude an influence of IFN inhibition by ncp BVDV early in infection on the outcome of the immune response. More studies are needed to better understand the mechanism of fetal transplacental infection as well as the mechanism of immunotolerance.

IMPACT OF HOST FACTORS ON THE OUTCOME OF REPRODUCTIVE DISEASE

From experimental studies attempting to infect other species, such as sheep and pigs, it is clear that BVDV is highly adapted for replication in the bovine species. Attempts to induce persistent BVDV infections by infecting pregnant sows at different stages of gestation have not produced consistent results. Although BVDV has been shown to induce persistent infection in lambs following in utero inoculation, congenital defects are more common following fetal infection than persistent infection. Lambs persistently infected with BVDV generally do not live for more than a few months. There have been no recognized differences between breeds of cattle when considering susceptibility to and severity of BVDV infection. Environmental factors such as cattle density, type of cattle operation (dairy or beef), type of housing, and biosecurity practices influence the severity and prevalence of disease in certain populations of cattle.

IMPACT OF VACCINATION ON THE OUTCOME OF REPRODUCTIVE DISEASE

Vaccination against BVDV has a positive impact on reproductive health. Both experimental and field

studies have proven that vaccination reduces the potential impact of fetal infections with BVDV. Understanding the strengths and limitations of vaccines has become important with the recognition of the significance of antigenic and genetic diversity in BVDV. Early vaccines were developed with little knowledge of their ability to provide fetal protection. According to 9CFR guidelines, for approval of BVDV vaccines in the United States, the vaccines were required to demonstrate reduced clinical disease in vaccinated animals but did not address the ability of the vaccine to prevent fetal infection. Recently, the USDA has developed guidelines for conducting fetal challenge experiments to obtain vaccine labels that can claim prevention of persistent infection and abortion due to BVDV infection. Vaccine efficacy in preventing the development of persistent infections is generally determined by experimental intranasal challenge of pregnant animals at 75 days of gestation with approximately 5×10^5 CCID₅₀/ml of BVDV (Cortese et al., 1998; Brock and Cortese, 2001). The status of the fetus and persistent infection are determined by detection of virus at least 75 days postchallenge (150 days of gestation).

The influence of antigenic diversity on fetal protection is evident by different rates of protection afforded by monovalent (type 1) vaccines when experimental challenge is done with type 1 or type 2 BVDV. Several field studies suggest that immunological protection against heterologous BVDV challenge may be incomplete with respect to fetal protection (Bolin et al., 1991; Kelling et al., 1990; Van Campen et al., 2000). The efficacy of killed vaccines in experimental studies has varied from 25–100%. In studies on evaluating the fetal protection efficacy of a modified-live vaccine, 88% and 58% levels of fetal protection were demonstrated in heifers immunized once with a commercial BVDV-1 vaccine and challenged at 75 days of gestation with type 1 or type 2 BVDV, respectively (Cortese et al., 1998; Brock and Cortese, 2001). Therefore, it was concluded that BVDV-1 vaccine was less likely to stimulate fetal protective immunity against BVDV type 2. To reduce the prevalence of persistent infections, it is important to promote routine vaccination as well as strict biosecurity practices to prevent the introduction and continued maintenance of cyclic BVDV infections.

For controlling BVDV, it is important to prevent fetal infections and the birth of calves that are persistently infected with BVDV. Eliminating fetal infections will correspondingly eliminate other reproductive losses. Prevention of fetal infections, and the

subsequent economic consequences, depend on reducing or eliminating exposure to BVDV and enhancing specific anti-BVDV immunity by vaccination (Sockett et al., 1997). Cattle persistently infected with BVDV are the primary source of virus spread within and between farms. Identifying and eliminating persistent infections should be a major focus when attempting to control and prevent BVDV. By eliminating persistently infected animals, the major source of virus capable of causing transient infections in pregnant dams and subsequent fetal infections is removed.

CONCLUSIONS

The reproductive consequences of BVDV infections are economically important because of the impact of both persistent and acute infections and of losses associated with producing animals persistently infected with BVDV. The primary consequence of reproductive infections is the occurrence of persistent infections. In addition, losses are due to a decrease in fertility and reproductive efficiency. Although the mechanism of persistent infections is known, the mechanism of reproductive pathogenesis is not known. Due to the capability of BVDV of crossing the placenta and infecting the fetus, vaccination is not considered to be an effective method for BVDV control.

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9

Immunity and Immunosuppression

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INTRODUCTION

Clinical manifestations of BVDV may include inapparent, acute, or persistent subclinical infections; fetal death and congenital abnormalities; wasting disease; severe acute disease (which may progress to hemorrhagic syndrome); or mucosal disease. The clinical outcome of infection depends on the immune status of the animal and time of infection. A complete understanding of the immune processes and immunogens of BVDV, as given in the next section, should be helpful in designing better preventive strategies for BVDV infections.

IMMUNE RESPONSES TO BVDV

BVDV strains vary in their tropism for bovine tissues. Hence, the effect of different strains of BVDV on the immune system also differs and may affect the type of disease exhibited. The natural route of transmission of BVDV is oronasal by contact with suspended droplets or mucus, although genital transmission may also occur. The virus first replicates in the nasal mucosa and tonsils from where white blood cells help spread the virus throughout the body by binding to surface receptors (Bruschke et al., 1998a, 1998b). Although the biochemical nature of BVDV receptors is not well understood, a 50 kDa protein is believed to be the viral receptor (Xue et al., 1997).

Pestivirus infections are associated with leukopenia, immunosuppression, and in some cases, hemorrhages. In general, the immunosuppressive properties of BVDV lead to a reduction in local defense mechanisms, thereby predisposing calves to other respiratory pathogens. Immune responses to BVDV may develop following vaccination, infection, exposure to cross-reactive pestiviruses, or by passively acquiring BVDV-specific antibodies from colostrum.

INNATE IMMUNE RESPONSE

The innate/natural (non-antigen-specific) immune response can influence the outcome of BVDV infection. BVDV can infect cells of the innate immune system (e.g., neutrophils, monocytes, macrophages, and dendritic cells) and affect their function (Potgieter, 1995; Glew et al., 2003; Lambot et al., 1998; Peterhans et al., 2002). Infection with BVDV may result in impairment of microbicidal, chemotactic, and antibody-dependent cell-mediated cytotoxicity of neutrophils (Potgieter, 1995). In monocytes, infection with cp BVDV may lead to apoptosis (Glew et al., 2003; Lambot et al., 1998). A 30–70% decrease in monocyte numbers may occur following infection of calves with virulent BVDV (Archambault et al., 2000). In vitro or in vivo infection of alveolar macrophages (AM) with BVDV may lead to decreases in phagocytosis, expression of Fc (FcR) and complement receptors (C3R), microbicidal activity, and chemotactic factors (Welsh et al., 1995; Liu et al., 1999; Peterhans et al., 2002). Infection of AM also causes increased LPS-induced (lipopolysaccharide) procoagulant activity, which can lead to bacterial colonization and may adversely affect the normal defense mechanism of the lung (Olchowy et al., 1997).

Cytokines can mediate the effects of both innate and specific immunity (Nobiron et al., 2001). Cytokines are small soluble proteins secreted by certain cells. They alter not only the function of cells producing them but also of other cells on which they might act. Tumor necrosis factor α (TNF- α) is a cytokine produced mainly by macrophages. It plays an important role in the activation of the immune response by modulating the production and activity of many other cytokines (Chase, 2004). Infection of AM with BVDV leads to a decrease in superoxide anion and TNF, enhanced nitric oxide (NO) synthesis in re-

sponse to LPS (Potgieter, 1995; Adler et al., 1994; Adler et al., 1997), stimulation of prostaglandin E2 synthesis (Van Reeth and Adair, 1997; Welsh and Adair, 1995), induction of IL-1 inhibitors (Jensen and Schultz, 1991), and a decrease in cytokine-induced chemotaxis (Ketelsen et al., 1979). Certain soluble factors released by infected monocytes and macrophages induce apoptosis when added to uninfected cells (Lambot et al., 1998; Adler et al., 1997). Apoptosis is the process that commits cells to programmed cell death to eliminate an infected cell and is believed to be the cause of lymphoid tissue pathology seen in mucosal disease and in disease caused by highly virulent BVDV (Liebler-Tenorio et al., 2003; Liebler-Tenorio and Ridpath, 2002; Stroffregen et al., 2000). Cytokines such as IL-2 and granulocyte-macrophage colony-stimulating factor enhance both humoral and cellular immune responses against BVDV and play a critical role in fetal-maternal interface by ensuring that pregnancy proceeds successfully (Graham et al., 1992). IL-1 is one of the endogenous pyrogens that act upon the hypothalamus to alter the regulation of body temperature.

Interferon (IFN) is the most important innate defense antiviral cytokine. Type I interferons are two serologically distinct proteins including IFN- α produced by phagocytes and IFN- β produced by fibroblasts. Viral infections, including BVDV infections, strongly signal the induction of type I IFN, which increases the cytotoxic potential of natural killer (NK) cells. Treatment of cells with high doses (10^4 units/ml) of human IFN- α prevented the replication of both ncp and cp BVDV in vitro while that with human IFN- γ , TNF- α or TNF- β did not (Sentsui et al., 1998). Infection of the fetus with cp BVDV leads to IFN production, which probably prevents the establishment of persistent infection. Infection of the fetus with ncp BVDV does not induce IFN- α production although it did induce abundant amounts of all IFN (α , β , and γ) in gnotobiotic calves (Charleston et al., 2001a, 2002). These responses were associated with depressed levels of transforming growth factor beta (TGF- β) in serum. These results indicate that the immunosuppression caused by ncp BVDV may not be associated with low interferon responses or elevated levels of TGF- β (Charleston et al., 2001a).

Antigen-presenting cells (APC; dendritic cells, macrophages, and monocytes) internalize the viral antigen and present it to T-helper cells with assistance from IFN- γ and IL-12. However, infection of APC with BVDV causes a reduction in Fc and C3 receptor expression, receptors that are required for

phagocytic activity (Welsh et al., 1995; Adler et al., 1996). It also reduces the ability of monocytes to present antigen to T-helper cells (Glew et al., 2003). Dendritic cells, the most important APC in the lymph node, on the other hand, were not affected in their ability to present antigen to T-helper cells or in surface marker expression (Glew et al., 2003).

HUMORAL IMMUNE RESPONSE

Although both active and passive humoral immune responses are protective, they differ in longevity and their ability to potentiate an immune response following a subsequent exposure. Antibody response to BVDV is detectable 2–3 weeks postinfection and may plateau in approximately 10–12 weeks postinfection (Howard et al., 1992). Humoral immunity, as detected by serum antibodies against BVDV, may result from an active immune response following an exposure to BVDV antigen (active immunity) or the ingestion of antibody present in colostrum (passive immunity). Three glycoproteins of BVDV (gp 53/E2, gp 48/E0, and gp 25/E1) induce neutralizing antibodies, with E2 protein being immunodominant (Bolin and Ridpath, 1990). Antibodies against several other viral proteins (115, 90, 48, and 25 kDa) have also been detected in some cattle (Bolin and Ridpath, 1990; Boulanger et al., 1991).

Colostrum antibodies

Antibodies do not cross the placenta of cattle as they do in humans. Thus, calves receive passive immunity by absorption, through the gastrointestinal tract, of immunoglobulins contained in the ingested colostrum. However, calves can absorb colostral antibodies only during the first 24–48 hrs of life when their gastrointestinal tract is permissive to the transfer of these molecules across the mucosal epithelium, a phenomenon called “the open gut.” The highest concentration of BVDV antibodies in colostrum occurs only in the first few days of lactation after parturition. After that, the colostrum changes to normal milk and the amount of antibodies decreases rapidly.

Passive antibodies play an important role in protection from BVDV infection in the neonatal calf. However, the presence of high concentrations of maternal antibody in animals may block the induction of active B-cell immune response to BVDV vaccination. This has led to the suggestion that vaccination should be administered when passively acquired antibodies are declining (Ellis et al., 2001). However, T-cell immune responses have been observed when calves were infected intranasally with BVDV in the presence of maternal antibodies

(Endsley et al., 2003; Ridpath et al., 2003), suggesting that vaccination in the face of maternal antibodies may be effective.

Passive immunity is not fail safe, however. Factors such as dose of challenge virus, poor nutrition, exposure to harsh weather, poor ventilation, transportation stress, and increased stocking density may affect the outcome of infection in the presence of colostral antibodies. In addition, antigenic variation among BVDV may result in incomplete protection, and colostrum may vary in quality and quantity of available antibodies. Nutrition and vaccination status of the dam will also affect the immune quality of colostrum. For example, vaccination of pregnant cows in the last month of pregnancy with killed vaccines will improve the amount of antiviral antibodies in colostrum. Vaccination during pregnancy with live, modified vaccines will also increase the amount of antibody in colostrum, but this type of vaccination is advised only when the dam has preexisting antibodies. Further, the benefits of colostrum are not limited to the action of passive antibodies; immune lymphocytes and macrophages present in colostrum are also of help. In this regard, fresh colostrum is better than frozen colostrums.

CELLULAR IMMUNE RESPONSE

Infection with BVDV results in mild (10–20% decrease) or severe lymphopenia (50–60% decrease) depending upon the strain of the virus (Brodersen and Kelling, 1999; Archambault et al., 2000). Cytotoxic T-lymphocytes (CD8+) are affected more than helper T-lymphocytes (CD4+) with little or no effect on circulating γ/δ T-cells (Brodersen and Kelling, 1999; Ellis et al. 1988). Depletion of CD4+ cells increases the period of virus shedding; that of CD8+ and γ/δ T-cells does not, indicating that CD4+ helper cells play a pivotal role in coordinating a cell-mediated response early in infection. These CD4+ responses are directed primarily at NS3 and E2 proteins (Lambot et al., 1997; Collen and Morrison, 2000; Collen et al., 2000; Collen et al., 2002) and also against the capsid protein (C), glycoprotein E^{ns}, amino-terminal proteinase (N^{pro}), and nonstructural protein 2-3 (NS2-3) (Collen et al., 2002).

Proliferation assays are an indirect measure of CD4+ responses to viral antigens. The cells of Th1 phenotype produce IL-2 and IFN- γ , but not IL-4 or B-cell stimulatory activity. In contrast, cells of Th2 phenotype express high levels of B-cell growth factor and IL-4 activity with relatively low levels of IL-2 and IFN- γ (Rhodes et al., 1999). IL-4 increases the expression of MHC II on B-cells and is also im-

portant for the growth and survival of Th2 responses. In cattle, unlike human and mouse systems, pregnancy has no observable shift in IL-4 pattern (Waldvogel et al., 2000).

The response generated by cp and ncp BVDV is different. For example, ncp viruses tend to shift the immune response toward the Th2 response and avoid the production of high levels of cell-mediated immunity (Lambot et al., 1997; Rhodes et al., 1999). cp BVDV, on the other hand, produces higher CMI response (Th1), along with up-regulation of IL-2 receptor (IL-2R) in response to increased levels of IL-2. The down-regulation of IFN- γ by acute ncp BVDV infection inhibits the cell-mediated response to *Mycobacterium bovis* that could result in the failure to identify cattle with tuberculosis (Charleston et al., 2001b). Delayed-type hypersensitivity is a cellular immune response that occurs approximately 18 hours after exposure to an antigen and is used as an assay for cell-mediated immunity. This response is characterized by induration and erythema at the site of antigen injection such as with the *Mycobacterium* antigen. After exposure to BVDV, the delayed type hypersensitivity to *Mycobacterium* is inhibited. Thus, BVDV causes general and nonspecific inhibition of cellular immune responses in cattle (Thoen and Waite, 1990) and may interfere with the diagnosis of bovine tuberculosis (Charleston et al., 2001b).

Proliferating CD8+ cytotoxic T-lymphocytes (CTL) produce IL-2 and IFN- γ , indicating a type 1 memory response in BVDV-seropositive cattle (Howard et al., 1992; Rhodes et al., 1999). Although fine mapping of BVDV CTL epitopes has not been done, computer predictions based on MHC I binding domains indicate that regions in the C, E^{ns}, E2, and NS2-3 are likely BVDV CTL epitopes (Hegde and Srikumaran, 1997).

IMMUNE RESPONSE OF PERSISTENTLY INFECTED ANIMALS

Persistent BVDV infections can arise in animals infected as fetuses. Persistently infected cattle are immunotolerant to the infecting BVDV isolate but may mount an immune response to heterologous BVDV. It has been demonstrated that heifers carrying PI calves developed BVDV antibody titers 5–10 times higher than those carrying non-PI calves (Brownlie et al., 1998). A number of studies have been done to understand the immunological defects in PI animals (Chase, 2004). The inability of ncp BVDV to induce IFN- α in the fetus is certainly one of the major immune evasion mechanisms that allow BVDV to establish persistence (Charleston et al., 2002).

ASSESSMENT OF IMMUNITY

HERD IMMUNITY

When assessing herd immunity, it is important to sample several animals or use a pooling strategy. The exact parameters of cellular and humoral immune responses that indicate protection against BVDV infection have not been determined. Veterinary practitioners routinely screen cattle herds for humoral immune response against BVDV to assess the effectiveness of vaccination programs. However, it is difficult to interpret serological profiles when only a single serum sample is tested. Because antibody titers resulting from natural infections usually exceed those generated from vaccination, uniformly high antibody titers within a herd or jointly housed group may indicate a recent BVDV outbreak. In addition, the presence of antibodies in fetal thoracic fluid or of precolostral antibodies in neonates is suggestive of fetal BVDV infection. Cattle that are not immunotolerant to BVDV antigens and are not sufficiently immunized can then be immunized with a BVDV vaccine containing a slightly different antigen. Enough individual variation exists among outbreak cattle that some animals may need booster injections to maintain high levels of herd immunity.

The serum neutralization (SN) test has been extensively used as a correlate of protective immunity. Since the SN test measures the total neutralizing activity of a serum sample or a body fluid, it represents a composite of neutralizing activity due to all classes of antibodies that have activity against surface proteins of BVDV. Some regions of viral proteins are highly conserved between members of pestiviruses, and others are variable between different members of the family. Most of the highly conserved regions are found in the inner part of the virion. Type-specific variable regions are present on the outer surface of the BVDV protein. Members of pestiviruses, such as BVDV, classical swine fever virus, and border disease virus often cross-react in SN tests (de Smit et al., 1999).

There are two major problems with BVDV serology as it is currently used: a lack of test standardization and the existence of antigenic variation among BVDV isolates. In an interlaboratory study of 14 U.S. diagnostic laboratories, SN titers for a single serum sample varied from 1:8 to 1:3642 (Vaughn, 1997). This variability may be due to technical expertise of the person performing the test and to virus strain, cell type and passage, and amount of virus used in the test. In one study, the SN titers varied by

5–10-fold with the use of different BVDV strains (Fort Dodge, 1999b) while in another study they varied from 2–250 fold (Fulton et al., 1997). Although ELISA tests hold the promise of negating many of the above variables, they have not been widely used because they suffer from a lack of correlation with SN titers, which appear to correlate relatively well with protective immunity against BVDV (Bolin and Saliki, 1996). At present, there are two recognized genotypes of BVDV (1 and 2) and several proposed subgenotypes. Although there is cross-reactivity between BVDV 1 and BVDV 2, cross-reactivity is significantly higher within a genotype than between genotypes (Jones et al., 2001). Most of the veterinary diagnostic laboratories in the U.S. now perform both type 1 and type 2 SN tests.

PROTECTIVE IMMUNITY

A large number of BVDV vaccines are available in the U.S. They are usually approved on the basis of their safety, their ability to induce serological response against the virus, and protection achieved in challenge protection experiments. In view of antigenic diversity of BVDV, the panel of challenge viruses should include both homologous and heterologous BVDV isolates from geographical areas where vaccines are to be administered. Inclusion of novel, antigenically different variants of BVDV in the challenge panel would also be of value.

BVDV has at least two genotypes (genotype 1 and 2) and two biotypes (cytopathic and noncytopathic). Despite widespread vaccination and the availability of a wide range of vaccines (Van Oirschot et al., 1999), BVDV remains a problem in most areas of the United States, raising the concern that evolution of the virus may occur under immunological pressure leading to the continued emergence of antigenic variation among BVDV isolates. With the recent recognition of genotype 2 BVDV, most vaccine manufacturers have now started to include both BVDV 1 and BVDV 2 in their vaccines. Although type 1 BVDV vaccines provide some protection against type 2 BVDV (Cortese et al., 1998; Fairbanks et al., 2003; Makoschey et al., 2001), a number of challenge studies have indicated that the best protection rates are obtained with the use of homologous BVDV vaccines (Potgieter, 1995; Fulton et al., 2003).

IMMUNOSUPPRESSION

Diverse clinical manifestations are associated with BVDV infection in cattle. Although a majority of

BVDV infections in immunocompetent cattle are transient and self-limiting, it is apparent that when infection occurs in the presence of other microorganisms, BVDV can contribute to a disease that becomes clinically evident (Baker, 1995). Both natural and experimental studies have demonstrated the relationship between BVDV and other infectious agents, suggesting that BVDV has the ability to induce immunosuppression in immunocompetent animals. Impairment of lymphocyte and neutrophil functions decreases in the number of circulating and tissue immune cells, and environmental and/or management stressors have been cited as contributors to immunosuppression (Potgieter, 1995).

BVDV AND SECONDARY INFECTIONS

Increased susceptibility to secondary infections is a consequence of BVDV-induced immunosuppression. BVDV increases susceptibility to secondary infections because lymphocytes from BVDV-infected animals have impaired memory responses to other antigens and BVDV (Lamontage et al., 1989). Some pathogens may induce disease alone, but the disease is enhanced in the presence of BVDV. In some cases, BVDV-induced disease can be enhanced by opportunistic organisms. Substantial data indicate that BVDV infection is important in multiple-etiology diseases. Bovine respiratory disease complex in feedlot animals and in intensively housed calves is an example of this type of multiple-etiology disease process.

It is debatable whether BVDV-induced immunosuppression or primary infection of the respiratory tract plays a major role in the bovine respiratory disease complex. Little experimental evidence exists establishing a primary role of BVDV in the bovine respiratory disease complex (Potgieter, 1997). To support a primary role, BVDV is frequently isolated from pneumonic lungs of cattle (Greig et al., 1981). In a study on respiratory disease outbreaks with multiple virus infections, BVDV was isolated from pneumonic cattle more frequently than any other virus (Richer et al., 1988). Suggestive evidence exists for certain BVDV isolates to be pneumotropic (Jewett et al., 1990; Potgieter et al., 1985). In an experimental infection study, endobronchial inoculation of two different isolates of BVDV resulted in interstitial pneumonia. However, when calves were challenged with *Mannheimia hemolytica*, more severe disease resulted with only one of the two isolates (Potgieter et al., 1985). In another study, certain BVDV isolates were shown to be able to induce severe respi-

ratory disease in gnotobiotic lambs (Jewett et al., 1990).

Although BVDV is frequently isolated from cattle with pneumonia, it is often present with other infectious agents, including bovine herpes virus-1 (BHV-1) (Biuk-Rudan et al., 1999; Greig et al., 1981), parainfluenza-3 virus (PI-3) (Dinter and Bakos, 1961; Fulton et al., 2000), bovine respiratory coronavirus (BCV), bovine respiratory syncytial virus (BRSV) (Brodersen and Kelling, 1998, 1999), *Mannheimia hemolytica* (Fulton et al., 2002), *Pasteurella multocida* (Fulton et al., 2002), *Mycoplasma bovis* (Martin et al., 1990; Shahriar et al., 2002; Haines et al., 2001), and *Hemophilus somnus*.

In addition to causing immunosuppression, BVDV may interact directly with pathogens to make them more virulent. Combined infection of bovine alveolar macrophages with BRSV and BVDV produces synergistic depression of alveolar macrophage functions (Liu et al., 1999). Concurrent infection with BVDV and BRSV in cattle causes more severe enteric and respiratory disease, and these animals shed higher concentrations of BVDV in their nasal secretions (Brodersen and Kelling, 1998). Reports from disease outbreaks and experimental studies have supported a role for synergism with BHV-1 also (Greig et al., 1981; Potgieter et al., 1984). An experimental infection study was performed for evaluating this synergism. Calves inoculated with BVDV 7 days prior to inoculation with the Cooper strain of BHV-1 developed severe clinical disease, with dissemination of BHV-1 into non-respiratory tissues, including intestinal and ocular tissues, as compared to calves inoculated with BHV-1 alone (Greig et al., 1981; Potgieter et al., 1984). These observations suggested that initial BVDV infection impaired the ability of calves to clear BHV-1 from the lungs and to contain BHV-1 at the local infection site.

The ability of BVDV to synergistically interact with pathogens does not appear to be confined to the respiratory tract either, since synergism between BVDV and enteric pathogens has also been reported (Kelling et al., 2002a; Woods et al., 1999). From experimental infection studies in calves, it has been observed that BVDV and rotavirus may interact in a synergistic manner to induce more severe clinical disease (Kelling et al., 2002a). Synergism between BVDV and transmissible gastroenteritis virus was demonstrated by generalized lymphocyte depletion throughout the lymphatic system and villous atrophy in the intestinal tract of experimentally infected pigs (Woods et al., 1999).

CLINICOPATHOLOGICAL ASSESSMENT OF IMMUNOSUPPRESSION

Although a majority of BVDV infections in immunocompetent, seronegative cattle are subclinical (Baker, 1995), pyrexia, mild inappetance, and a decrease in milk production can be detected on close examination. In clinical infections in cattle, hematologic abnormalities such as thrombocytopenia and leukopenia are frequently reported (Archambault et al., 2000; Bolin et al., 1985; Ellis et al., 1998; Kelling et al., 2002a, 2002b; Walz et al., 2001). Decreases in white blood cells in the peripheral circulation (quantitative disorder or leukopenia) as well as alterations in function of these cells (qualitative disorder) in the peripheral circulation or in tissues are the basis for BVDV-induced immunosuppression. Although both qualitative and quantitative white blood cell disorders may be observed in acute infections, only qualitative disorders are observed in PI cattle (Brown et al., 1991; Muscoplat et al., 1973; Roth et al., 1986).

A transient leukopenia occurs in most cattle acutely infected with BVDV. The very first description of BVDV by Olafson et al. (1946) demonstrated leukopenia as a finding in both naturally and experimentally infected cattle. White blood cell concentrations decrease to various degrees during BVDV infection. White blood cell count for healthy cattle is 4,000–12,000 leukocytes/ μ l with a mean of 8,000 leukocytes/ μ l (Kramer, 2000), and the leukocyte concentration is higher in neonatal calves (Tennant et al., 1974). By definition, leukopenia is a decrease in white blood cell concentration below the normal level. Early reports have identified mild reductions in white blood cell concentration in calves with minimal evidence of clinical disease. There seems to be no difference between cytopathic and noncytopathic biotypes as far as production of leukopenia is concerned. After intravenous challenge with a cytopathic BVDV, the mean white blood cell concentration decreased from 7,850 leukocytes/ μ l to 5,050 leukocytes/ μ l at 4 days after infection (Bolin et al., 1985). In an experimental infection with a noncytopathic isolate of BVDV, a mild leukopenia was observed on days 3, 5, and 7 after infection (Ellis et al., 1988) with leukocyte concentrations of <5,000 leukocytes/ μ l. The typical time frame for depression in white blood cell concentrations is between 3 and 12 days after infection (Bolin and Ridpath, 1992; Ellis et al., 1998; Kelling et al., 2002a, 2002b; Walz et al., 1999).

Differences in the degree of leukopenia are associated with the virulence of the virus. Although severe acute disease has been associated only with type 2 BVDV, genotype is not a determinant for virulence. Though some type 2 strains cause severe acute disease, infection with other BVDV 2 strains results in subclinical to mild disease under natural and experimental conditions (Marshall et al., 1996; Walz et al., 2001; Liebler-Tenorio et al., 2003). Kelling et al. (2002b) evaluated five different isolates of BVDV 2 and found that although all five induced leukopenia, the highly virulent isolates induced a significantly more severe lymphopenia than the less virulent isolates. Further study revealed that virulence correlated with depression in lymphocyte counts. In an experimental infection, a highly virulent strain of BVDV 2–induced depression in lymphocyte counts of greater than 80% from preinoculation levels; a less virulent BVDV strain induced depressions of less than 50% (Liebler-Tenorio et al., 2003).

Not only are absolute counts affected by virus virulence, but duration of leukocyte depressions also correlates with virulence (Liebler-Tenorio et al., 2003; Walz et al., 2001). In a study involving two type 2 isolates and one type 1 isolate, the more virulent type 2 isolate induced a more severe leukopenia of a longer duration than the less virulent type 2 isolate and the type 1 isolate (Walz et al., 2001). Differences have also been reported in the type of affected leukocytes following experimental challenge. For example, neutropenia was observed as the major hematologic abnormality with some BVDV isolates (Walz et al., 2001; Hamers et al., 2000; Archambault et al., 2000), and lymphopenia was observed with some others (Bolin et al., 1985; Kelling et al., 2002b). In yet other studies, reduction in both lymphocytes and neutrophils has been documented (Ellis et al., 1988, 1998).

Unlike acutely infected animals, the PI calves have normal numbers of leukocytes and lymphocytes (Bolin et al., 1985; Larsson et al., 1988) but the proportion of lymphocyte subpopulation might change. For example, PI calves have an increased proportion of B-cells and diminished numbers of lymphocytes not identified as B-cells or T-cells (null cells) (Larsson et al., 1988). The mechanism of BVDV-induced leukopenia is currently unknown. Several possibilities exist, including immune system removal of BVDV-infected immune cells, destruction of immune cells by BVDV, and increased trafficking of immune cells into tissue sites of viral replication.

BVDV-INDUCED IMMUNE ORGAN DYSFUNCTION

Specific changes in immune system function occur with acute BVDV infection. As described above, all types of immune cells are infected by BVDV and their functions affected.

Effect of BVDV on bone marrow

Viral antigen is present in megakaryocytes and myeloid cells of BVDV-infected cattle (Spagnuolo et al., 1997). Bone marrow-derived macrophages can be infected in vitro with BVDV, and after infection, they release type I interferon (Adler et al., 1997). It has been suggested that type I interferon might prime the oral cavity and gastrointestinal tract to decrease NO production and to induce apoptosis in response to LPS (Alder et al., 1997). This biochemical pathway may be the basis of mucosal disease caused by BVDV. In acute BVDV infection, the development of lesions in lymphoid tissue is a function of BVDV replication and host reaction to infection (Liebler-Tenorio et al., 2002).

Effect of BVDV on thymus

Thymus plays an important role in the maturation of CD4+ and CD8+ lymphocytes. Thymus is a central lymphoid organ and all lymphocytes that enter thymus get selected based on molecules expressed on their surface. Lymphocytes that strongly recognize self-antigens are negatively selected in the thymus. Only 1–2% of lymphocytes that enter the thymus mature. The remaining lymphocytes undergo clonal deletion during the selection process. It is from these selected cells that BVDV-specific T-cells are generated. Infection with BVDV significantly decreases thymocyte function (Marshall et al., 1994). Like all lymphoid tissues, there are lesions (such as depletion of lymphocytes) but viral antigen is not detected except in vascular walls only (Liebler-Tenorio et al., 2002).

Effect of BVDV on Peyer's patches

BVDV significantly alters T-cells in Peyer's patches. After BVDV infection, there is significant depletion of lymphocytes in Peyer's patches, and cattle in terminal stages of mucosal disease have extensive loss of lymphocytes in the gut-associated lymphoid tissues (Figure 9.1). The tips of the domes are depleted of lymphocytes and the epithelia of the follicles are invaginated. The number of CD4+ cells decreases in the follicles and the number of lymphocytes in inter-follicular areas is also reduced (Liebler et al., 1995). B-lymphocytes are also depleted in lymphoid folli-

cles leading to decreased size of the follicles (Liebler et al., 1995).

Effect of BVDV on spleen

Small arterioles in the spleen are surrounded by periarteriole lymphoid sheaths that contain CD4+ and CD8+ T-cells. Masses of B-cells called lymphoid follicles are attached to T-cell sheaths. Both cytopathic and non cytopathic biotypes of BVDV infect spleen cells and the virus has been isolated from spleen of cattle that die from BVDV (McClurkin et al., 1985 and Bolin et al., 1987). In the initial phase of BVDV infection, virus is present but no lesions occur. However, lesions develop later in infection and antigen disappears in about 2 weeks.

IMMUNOLOGICALLY PRIVILEGED SITES

In immunologically privileged sites, the immune response is inaccessible or suppressed. In these organs, the immune response is contained to prevent inflammation that could be destructive to the virus-infected tissues.

TESTES

BVDV infection of breeding bulls leads to a decrease in semen quality, although the virus may or may not be detectable in the seminal ejaculate (Fray et al., 2000). In one study, BVDV was isolated from raw, unprocessed, and diluted extended semen (Kirkland et al., 1991) with titers ranging from 5–75 TCID₅₀/ml. Testes can be a site of persistent BVDV infection in non-viremic bulls (Niskanen et al., 2002; Givens et al., 2002). In calves infected with BVDV, seminiferous tubules are lined with sertoli cells and intratubular giant cells (Binkhorst et al., 1983). Studies done in other species (such as humans) indicate that testicular and epidymal lymphocytes express T-cell markers (Yakirevich et al., 2002). Studies to investigate immune responses to BVDV in testes of cattle are sorely needed.

OVARIES

Cattle persistently infected with BVDV contain viral RNA and antigen in ovarian tissues (Booth et al., 1995). In cattle acutely infected with ncp BVDV, the levels of gonadotrophins and sex strands are modulated (Fray et al., 2002). Bovine follicular cells and oocytes are permissive to BVDV infection, and acute infection decreases estradiol secretion (Fray et al., 2000). The replication of BVDV in ovaries causes ovarian dysfunction and reduced fertility (Shin and Aclaud, 2001).

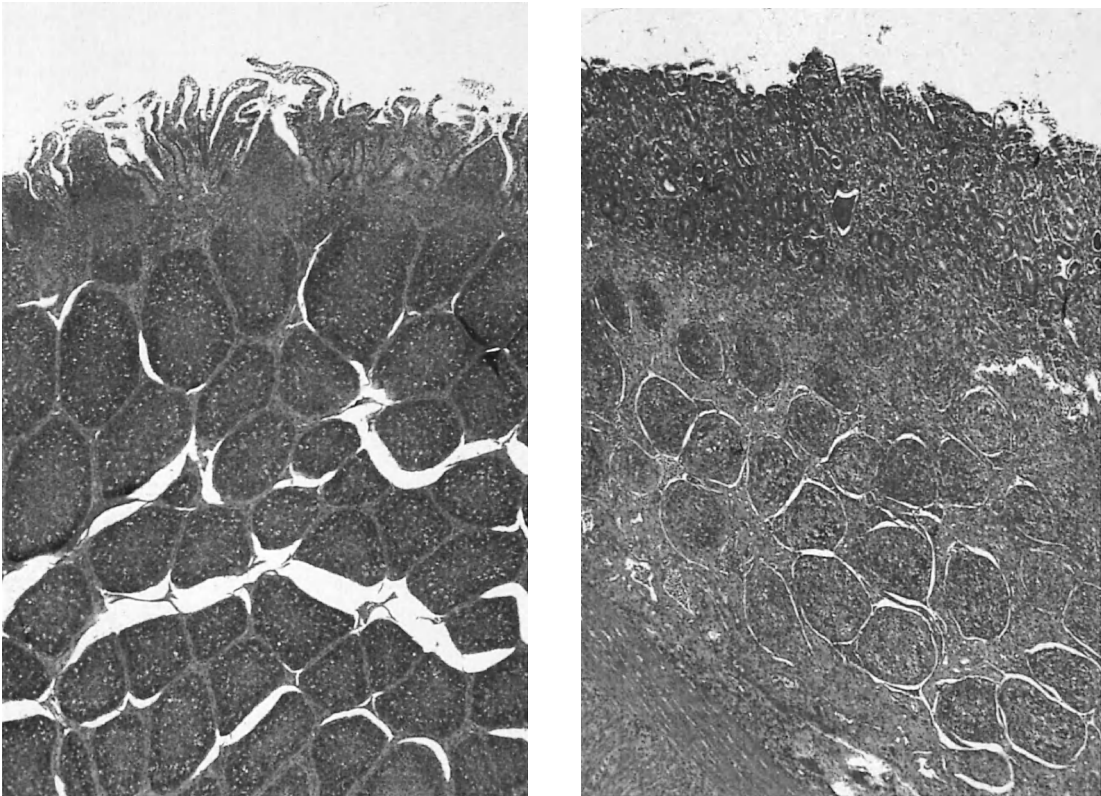


Figure 9.1. **Left.** Histologic section of ileum from an uninfected calf. **Right.** Histologic section of ileum from a calf 12 days after infection with BVDV 890 (BVDV type 2). Note the depletion of lymphoid follicles.

CNS

Calves born to BVD-infected cows develop CNS disorders at various times after birth, including nystagmus, eye disorders, gait disorders, and tremors. Congenital malformations occur during the 3rd to 5th months of gestation. Cerebellar hypoplasia is the most common lesion and is also associated with hydrocephalus and hydraencephaly. BVDV infection is accompanied by lens opacity and retinal atrophy. Blindness in calves is associated with no blinking reflex. BVDV-infected animals develop demyelination of the spinal cord, which is consistently present in the white matter (Hewicker-Trautwein et al., 1995).

SUMMARY AND CONCLUSIONS

Bovine viral diarrhea virus continues to be a major problem for the cattle industry worldwide. The virus is intimately associated with immunological disarrangement because it infects the immune cells of the body, including the antigen-presenting cells/

macrophages, and B- and T-cells. The virus has an impressive ability to evade immune recognition and elimination, which is best exemplified by its ability to infect the developing fetus, induce immunotolerance and persistent infection, and thus perpetuate itself in the herd for future generations of calves. In addition, BVDV may persist in immunologically privileged sites, such as ovaries and testicles, and thus is present in the germ plasm. Antigenic and genetic variations are also hallmarks of BVDV due to the extreme plasticity of the virus, and this variation affects immune system recognition of the virus.

Vaccines developed for BVDV have existed for several decades and the use of BVDV vaccines is fairly common. In spite of their widespread usage, BVDV vaccines appear to offer incomplete fetal protection due to the inability to achieve total protection against viremia. Understanding the methods by which BVDV interacts with the bovine immune system should provide insight into the pathogenesis of the virus, which may ultimately lead to the development of more effective vaccines. Thus, the key

areas of future research with respect to BVDV and the immune system are

- Understanding the roles of different components of the immune system in protection as they relate to vaccine development and evolution of BVDV genetic and antigenic diversity
- Determining the mechanisms of BVDV-induced immunosuppression and immunotolerance

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10

Hosts

Trevor R. Ames

INTRODUCTION

Pestiviruses of domestic animals include bovine viral diarrhea virus (BVDV) of cattle, border disease virus (BDV) of sheep, and classic swine fever virus (CSFV) of pigs. The latter virus is also known as hog cholera virus (HCV). These viruses were initially found to be associated only with their species of origin but are now recognized to infect numerous other animal species indicating they are not species-specific. This chapter focuses on BVDV and hosts other than cattle in which infection results in disease, virus isolation, or seroconversion. Additionally, cell types supporting the growth of BVDV in vitro are also discussed.

Concern exists over non-bovine hosts of BVDV for a number of reasons. In many agricultural situations, cattle and other wild and domestic ruminants share the same pasture, range, and water source. Transmission of virus from non-bovine hosts to cattle may affect control programs and/or productivity in cattle herds. Similarly, spread of BVDV from cattle to sheep, goats, and wild ruminants may affect the productivity of domestic animals or the health of wildlife populations. In addition, infection of species such as swine with BVDV may interfere with HCV control programs through false positive test results. In Europe, formal monitoring of wildlife for domestic animal pathogens (such as pestiviruses) is carried out in some countries (Frolich et al., 2002). This type of surveillance would be needed should control programs for pestiviruses be considered in North America.

An additional concern worldwide is the possible BVDV contamination of biologics (particularly modified live virus vaccines) used in non-bovine hosts. Biologic manufacturers may use BVDV-contaminated fetal bovine serum in vaccine production as a result of inadequate testing or improper

quality control standards. Pandemics may start when a pathogen “jumps” from one species to another. The parvovirus jump from feline to canine hosts may be an example of such a pandemic occurring due to contaminated biologics.

DISEASE SYNDROMES IN NON-BOVINE HOSTS DUE TO NATURAL BVDV INFECTION

Reports of disease resulting from natural infection with BVDV in species other than cattle are infrequent. BVDV was initially isolated from litters of dying piglets as well as from stillborn pigs in 1988 (Terpstra and Wensvoort, 1988). In a subsequent report, these authors reported a series of 19 naturally occurring and 8 experimentally induced incidences of congenital BVDV infections in pigs (Terpstra and Wensvoort, 1991). The disease was restricted to the involved litter without any horizontal transmission. The signs resembled those of CSF with pigs showing signs of infection at birth and dying by 4 months of age. Postpartum infections, in contrast, usually resulted in subclinical disease. Rarely, congenitally infected pigs may survive and become lifelong carriers of the virus, shedding the virus in urine, semen, and pharyngeal fluid (Terpstra and Wensvoort, 1997).

Outbreaks of a disease resembling border disease in sheep have been reported as a result of BVDV being spread from persistently infected calves (Carlsson, 1991). BVDV has also been isolated from congenitally infected kids and lambs showing enteric symptoms resulting in death in the first week of life (Nettleton et al., 1980). BVDV was also detected in a stillborn alpaca by reverse transcription-polymerase chain reaction (RT-PCR) (Goyal et al., 2002). The virus was subsequently isolated and typed as BVDV type 1b. BVDV may have been the cause of death for this alpaca although no gross or histologic lesions

were found. Mucosal disease-like lesions have been described in a free-ranging white-tail deer from which a cytopathic BVDV was isolated (Ludwig and McClurkin, 1981). BVDV type 1a was isolated from a free-ranging female mule deer in Wyoming (Van Campen et al., 2001). The deer was emaciated, weak, salivating, and had lung abscesses from which *Arcanobacter pyogenes* was cultured.

DISEASE SYNDROMES IN NON-BOVINE HOSTS DUE TO EXPERIMENTAL BVDV INFECTION

BVDV has also been shown to produce disease in a range of domestic animals following experimental challenge. Oral-intranasal infection with field strains of BVDV in pregnant gilts produced intrauterine infection in 1 of 20 gilts, with an additional 3 gilts having reduced fetal numbers compared to corpora lutea and 2 gilts being barren (Stewart et al., 1980). Intranasal and subcutaneous administration of a vaccine strain of BVDV (Oregon C24V) in pregnant sows in the second trimester of gestation produced asymptomatic seroconversion during second and third trimesters of pregnancy (Kulscar et al., 2001). Some of the piglets born at term had clinically apparent disease consistent with that caused by CSFV, indicating transplacental infection. Fifty-seven percent of the piglets died by 60 days of age, but neither the sows nor their progeny shed the virus. Similarly, a BVDV or BDV that was present as a contaminant in a CSF vaccine induced congenital pestivirus infection in piglets born on eight swine farms utilizing the vaccine (Wensvoort and Terpstra, 1988).

Experimental, intranasal challenge of 2-month-old pigs with various doses of type 1 and type 2 BVDV was performed to evaluate the pathogenicity of the two BVDV isolates in a swine model (Paul et al., 1999). Neither virus induced clinical signs although both were reisolated antemortem and postmortem. Type 2 BVDV isolate that was experimentally more pathogenic in calves failed to produce disease in pigs, indicating the virulence was species-specific. These reports support the arguments that congenital infection of pigs with BVDV may result in disease but postnatal infection rarely produces clinical signs (Terpstra and Wensvoort, 1988; Terpstra and Wensvoort, 1991).

Transplacental infection of ovine fetuses with BVDV has been studied extensively in sheep. BVDV can cross the placenta and produce fetal lesions within 10 days postinfection (Hewicker-Traut-

wein, 1994). Type 2 BVDV has also been shown to produce congenital lesions in sheep fetuses (Scherer et al., 2001). BVDV infection of pregnant ewes, like BDV infection, results in abortions, stillbirths, unviable lambs, and viable lambs that are both virus-positive and virus-negative (Scherer et al., 2001). Because of the similarity of BVDV to BDV, and the behavior of the BVDV in the pregnant ewe, experimental challenge of vaccinated ewes with BVDV has been used to evaluate protective immunity, including fetal protection, afforded by various BVDV vaccines (Brushke et al., 1996).

BVDV can produce pulmonary lesions in 6–8-month-old lambs following intranasal and intra-bronchial challenge (Meeffhan et al., 1998). New-born lambs and kids challenged with BVDV isolates most consistently have CNS lesions (Jewett et al., 1990; Loken et al., 1990). Deer and elk have also been experimentally challenged with BVDV to determine whether these wild ruminants are capable of becoming infected and shedding the virus (Tessaro et al., 1999; van Campen et al., 1997). Both deer and elk can be infected, shed the virus, and seroconvert to the virus (Terpstra and Wensvoort, 1997; van Campen and Williams, 1996). In addition, lateral transmission to nonchallenged in-contact animals has been demonstrated with elk (Tessaro et al., 1999). Experimental infection of pregnant does with a deer isolate of BVDV resulted in mummified fetuses, stillborn fawns, and live fawns (Ludwig and McClurkin, 1981).

VIRUS ISOLATION AND SEROCONVERSION IN NON-BOVINE HOSTS

As stated in the previous section, BVDV has been isolated from sheep, goats, and pigs undergoing natural infections (Carlsson, 1991; Pratelli et al., 1999; Terpstra and Wensvoort, 1988). BVDV has also been isolated from German roe deer (Tessaro et al., 1999), Scottish deer (Fischer et al., 1998), and exotic captive ruminants (Nattleton, 1990). Isolation of pestiviruses from a variety of ruminants other than cattle indicates that many species not yet studied may harbor them (Doyle and Heuschele, 1983). Recently, persistent infection has been documented in a family of captive mousedeer indicating that this phenomenon also occurs in cervids (Grondahl et al., 2003). This finding has significant implications for eradication campaigns.

Serological surveys also provide useful information on which species may become infected with BVDV and the seroprevalence within those species.

A survey of 1,133 dairy cows, 3,712 ewes, and 1,317 adult pigs in Norway found that 18.5% of cattle, 4.5% of sheep, and 2.2% of pigs were seropositive for BVDV (Loken et al., 1991). Of the swine that tested positive, all had higher antibody titers against BVDV than against CSFV indicating that exposure was likely to BVDV rather than to CSFV. No such comparisons were made between BVDV antibody titers in the seropositive sheep, so it is unclear whether these titers are a true reflection of BVDV seroprevalence. In a study of ruminants in Namibia, neutralizing antibodies to BVDV were found in 58% of cattle, 14% of sheep, and 4.6% of goats (Depner et al., 1991). Again no attempt was made to differentiate the pestivirus species causing these high serologic titers. In a similar study in Northern Ireland, 5.3% of ewes and 30.4% of flocks were found positive to BDV (Graham et al., 2001). Upon further testing it was found that all sheep had higher titers to BVDV type 1 than to BDV. In addition, the only pig found seropositive of 680 tested for HCV actually had higher titers to BVDV type 1 than to CSFV. These studies point out the importance of determining which pestivirus a group of animals is infected with or is responding to serologically, especially when control programs are being considered. Serologic evidence also exists for pestivirus infection of goats (Nattleton, 1990).

Serologic surveys of approximately 50 captive and free living ruminant species distributed within the families of Camelidae, Cervidae, Antilocapridae, and Bovidae have shown presence of pestivirus antibodies (Loken, 1995; Nattleton et al., 1980). Seventeen different species of African wildlife have tested positive for antibodies to BVDV (Hambli and Hedger, 1979). In North America, 2% of pronghorn antelope and 31% of American Bison have antibodies to BVDV (Stauber et al., 1980; Taylor et al., 1997). Seropositivity in caribou herds may range from 41–100% ((Dieterich, 1987; Elazhary et al., 1981; Stuen et al., 1993; van Campen and Williams, 1996) but with moose the seroprevalence ranges from 12–18% (Kocan et al., 1986; Thorsen and Henderson, 1971). This suggests that wild ruminants that are part of large herds, such as caribou and bison herds, may have higher seroprevalence than more solitary wild ruminants such as the moose or those that travel in smaller groups such as the antelope (van Campen and Williams, 1996).

Two percent of llamas in Argentina have antibodies to BVDV indicating that imported llamas may also harbor this virus and may transmit to cattle herds if in close proximity (Puntel et al., 1999).

However, the seroprevalence of BVDV antibodies in deer has not been shown to be affected by cattle population densities in deer habitats (Frolich, 1995). Sequence analysis of BVDV isolated from roe deer found the isolate to be genetically unique compared to other BVDV isolates (Frolich and Hofmann, 1995). It is likely that BVDV-strains circulate independently within deer populations, with transmission rarely occurring between deer and domestic livestock (Fischer et al., 1998). Serologic evidence for BVDV infection in rabbits has been shown with 40% of free ranging rabbits in Germany having antibody titers to BVDV in one report (Frolich and Streich, 1998). None of the rabbit spleens examined in this study were positive for BVDV.

Some evidence exists for BVDV infection in humans (Giangaspero et al., 1993). The seroprevalence of BVDV antibodies in one study of human subjects in Zambia ranged from 14.75% in healthy non-HIV-infected humans to 19.2% in HIV-infected individuals with chronic diarrhea. The increased seroprevalence in this latter group was statistically significant leading the authors to speculate that some interaction between HIV and BVDV may exist. There are a number of concerns, however, that arise from this report:

- No other reports have documented seroconversion to BVDV in humans who work with BVDV or BVDV-infected animals.
- BVDV has not been successfully grown in primate cells, raising doubts about the potential for infection and seroconversion in primates.
- Antibody titers against other pestiviruses were not determined, making it difficult to conclude whether the antibodies detected were specifically against BVDV
- The researchers failed to provide evidence that no reagents used in their investigation were contaminated with BVDV or BVDV antibodies.

CELL LINES SUPPORTING THE GROWTH OF BVDV AS POSSIBLE INDICATORS OF HOST RANGE

In a large survey of 41 common cell lines, cells of cattle, sheep, goat, deer, bison, rabbit, domestic cat, and swine origin were found capable of supporting BVDV growth. A subpopulation of these cell lines including those of cattle, sheep, goat, deer, bison, rabbit, and domestic cat origin were found to be contaminated by BVDV using immunohistochemical and polymerase chain reaction procedures (Bolin et al., 1994). This study not only points out the abil-

ity of BVDV to grow in a variety of cell lines but also the potential for it to contaminate cell lines inadvertently. Interestingly, although the virus is capable of infecting many cell lines, cell tropisms do exist, and the viral glycoprotein E2 has been shown to play a role in this cell tropism (Liang et al., 2003). As previously discussed, researchers and diagnosticians must control for possible cell line contamination with BVDV or the results of investigative studies will be compromised.

CONCLUSIONS

The reports of naturally occurring disease indicate that pigs and a variety of domestic and wild ruminants can be infected with BVDV. The virus has been shown to produce congenital infection in pigs, sheep, and goats. In some cases, congenital infection results in persistently infected animals that can be a significant source of viral transmission. Wild ruminants are known to be susceptible to acute infections with BVDV, and limited evidence suggests that cervids may also undergo congenital infections leading to persistent infection (Grondahl et al., 2003). This suggests a new potential for this virus to be shed in large amounts from wild ruminants that could then infect cattle. Current evidence suggests that wild ruminants may serve as a transient source of virus while undergoing acute infections and may possibly be a more prolonged source of virus from persistently infected animals. This would be of greatest concern where these animals are in contact for prolonged periods of time, as opposed to transient fence line contact.

The role of species from which the virus has never been isolated but in which seroconversion has been observed is not clear. The significance of in vitro growth of BVDV in certain cell lines without infection in whole animals is also not known. It is unlikely, however, that these species would be viremic for significant periods of time and thus are unlikely to shed BVDV in the environment. Based on the previous discussion, it seems that non-bovine domestic ruminants and swine remain the greater concern for disrupting management attempts to control the diseases caused by BVDV in cattle.

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11

Interactions of Virus and Host

John D. Neill

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is a ubiquitous pathogen of ruminants that is often associated with severe economic losses. Understanding these viruses, particularly at the cellular and molecular levels, is important to develop new vaccination and treatment strategies. The events that transpire following infection of the host animal are only now beginning to become clear. Upon entry into a susceptible host cell, the virus replicates utilizing viral as well as cellular proteins and machinery. Our understanding of BVDV replication comes from studies of BVDV directly or is extrapolated from studies of classical swine fever virus (CSFV, known previously as *hog cholera virus*) and other members of the Flaviviridae, particularly hepatitis C virus (HCV). Most proteins encoded by BVDV, CSFV, and HCV, as well as RNA structural motifs, are considered functionally equivalent (Rice, 1996; Branza-Nichita et al., 2001). The two exceptions are the N^{pro} and E^{ns} proteins that are unique to pestiviruses. This chapter describes functions provided by the host cell that are absolutely required for virus replication, including protein translation, protein modification, and transport and release of progeny virus. Results from studies of other pestiviruses and flaviviruses are discussed in context of BVDV and will be considered common to all viruses unless stated otherwise.

RECEPTOR AND VIRUS ATTACHMENT

Arguably, the single most important event in the life cycle of a virus, the “make or break point,” is the attachment of the infectious virus particle to the surface of a susceptible cell. If this does not occur, downstream events such as uptake, release of genome, replication of viral RNA(s) and translation of viral proteins cannot take place. Binding of the

virus to the cell surface has been shown in a number of systems to involve the recognition of specific cellular molecules that are embedded in, or intimately associated with, the cell's plasma membrane. These specific cellular molecules are usually proteins, although many examples of viruses binding to carbohydrate moieties are known (Jackson et al., 1996; Hilgard and Stockert, 2000; Martínez and Melero, 2000; Dehecchi et al., 2001; Terry-Allison et al., 2001). Virus receptors are normal components of the plasma membrane and serve diverse functions such as internalization of ligands, cell signaling, and cell-to-cell interactions (Mendelsohn et al., 1989; Wykes et al., 1993; Colston and Racaniello, 1994; Roivainen et al., 1994; Agnello et al., 1999). The types and nature of these receptor molecules affects both the host and tissue tropism of the virus. If the receptor molecule of an organism is sufficiently different from that of the normal host, infection will not occur. Additionally, the receptor molecule(s) may be expressed in only a subset of cell types in the host, only at specific points in the cells cycle, or on specific surfaces of polarized cells (Compans, 1995). Any of these factors would limit the infection to specific cells or tissues. This in turn gives rise to the characteristic disease symptoms, lesions, and pathology of the viral infection.

CELLULAR FACTORS

Several groups have studied the interaction of BVDV and the cell surface. Initial studies involved the production of monoclonal antibodies (Mab) directed against cell surface epitopes. Teyssedou et al. (1987) reported the production of a Mab that was reactive against a protein on the surface of Madin-Darby bovine kidney (MDBK) cells. When attached, this Mab completely prevented infection by bovine enterovirus-3 (BEV-3) strain 240A and par-

tially inhibited infection by BEV-2 and BVDV. The mechanism behind this incomplete inhibition was unclear and probably did not represent an antibody reactive against the true receptor molecule. This was followed shortly by a report by Moennig et al. (1988) of a bovine-specific Mab that prevents infection of bovine cells with a number of cytopathic strains of BVDV (cp BVDV) while not preventing infection with CSFV, bovine herpesvirus-1, parainfluenza-3, and pseudorabies virus. Based on these findings, the authors suggested a single receptor molecule for BVDV. Further analysis of the protection afforded by this Mab, using the more sensitive immunoperoxidase method (Collett et al., 1989), revealed small numbers of infected foci in Mab-treated MDBK cultures, indicating the presence of multiple cell surface receptors for BVDV.

Another type of Mab, anti-idiotypic antibodies, were produced to study specific virus-receptor interactions (Xue and Minocha, 1993; Xue and Minocha, 1996, Xue et al., 1997). Anti-idiotypic Mabs were raised against neutralizing Mabs that bound the viral protein anti-gp53 (E2). These anti-idiotypic Mabs thus mimicked the viral epitopes presumed to be associated with binding of the virus to the cellular receptor. The anti-idiotypic Mabs bound to the surface of MDBK cells in a manner similar to that of the virus and inhibited infection by BVDV. A 50 kDa protein was identified in immunoprecipitation experiments as the target of these Mabs (Xue and Minocha, 1993; Minocha et al., 1997). Xue et al. (1997) determined that this receptor molecule was protein in nature because protease treatment of cells resulted in concurrent loss of virus binding to the cell surface. Glycosidase treatment of cells prior to infection was used to demonstrate that glycosylation of the receptor protein was not necessary for binding of virus. The receptor molecule was found on a number of different cell types, both susceptible and nonsusceptible to infection with BVDV (Xue and Minocha, 1993; Xue and Minocha, 1996), indicating a protein of conserved function. The receptor protein was recognized by BVDV on the surface of porcine cells, based on the resulting productive infection. However, the replication of the virus was slower, with progeny virus levels eventually reaching those produced by bovine cells. The difference was probably not a result of cell surface binding and uptake but rather in functions related to replication.

Three additional Mabs (Moennig et al., 1988) that bound to the surface of cells susceptible to infection with BVDV were characterized by Schelp et al. (1995). These Mabs bound to the surface of cultured

cells as well as leukocytes freshly isolated from the blood of cattle and blocked infection with BVDV to varying degrees. When used in immunoprecipitation experiments, all precipitated cellular proteins of 60 and 93 kDa. In follow-up experiments (Schelp et al., 2000), one of these Mabs, BVD/CA 26, recognized a protein consisting of 28 and 56 kDa subunits that, under nonreducing conditions, formed multimers of approximately 200 kDa. The 56 kDa subunit was shown to bind to F-actin, giving some insight into its possible biological function. It is unknown why there was a discrepancy in protein sizes between the two reports. It is also unknown if the 56 kDa protein described here and the 50 kDa protein described by Xue and Minocha (1993) are the same or related proteins.

A different approach examining BVDV binding and uptake was taken by Flores and Donis (1995). They generated an MDBK cell line that was resistant to infection by BVDV. This mutant cell line, CRIB, was developed by infection of a susceptible monolayer of cells with a cytopathic strain of BVDV (Singer strain) and culturing of the survivors. The resulting culture contained no virus (infectious or defective) as determined by cocultivation, animal inoculation, immunofluorescence, western and northern blots, and reverse-transcription polymerase chain reaction (RT-PCR). Infection of CRIB cells with 24 additional strains of BVDV did not result in a productive infection as determined by expression of viral proteins. Transfection of viral RNA or virions in the presence of polyethylene glycol (PEG) did result in a productive infection as measured by immunofluorescence, indicating CRIB cells were defective at virus entry and not at a postuptake function. However, CRIB cells were not resistant to infection by 10 other bovine viruses, indicating that the block of BVDV replication was specific and was not a general antiviral activity.

In additional studies, Flores et al. (1996) reported that resistance of CRIB cells to infection with BVDV was blocked at virus entry and suggested that a cell membrane function that was important in virus uptake following viral attachment was mutated or missing. Using PEG-mediated virus uptake in these cells, the authors concluded that while CRIB cells bound saturating levels of virus, entry was blocked by a defect in endocytosis. This was further demonstrated by blockage of PEG-mediated uptake of virus using inhibitors of endocytosis and endosomal acidification.

Characterization of the cell surface molecule bound by the E2 envelope provided evidence that the low density lipoprotein receptor (LDLR) was the

E2 receptor molecule as well as the mediator of endocytosis of the attached virus particle protein (Agnello et al., 1999). The LDLR is a cell surface endocytic receptor that mediates the uptake of extracellular ligands into the cell (May et al., 2003). Three lines of evidence support the LDLR as a BVDV receptor: complete inhibition of endocytosis by anti-LDLR antibodies, inhibition of endocytosis by phenylarsine oxide (an inhibitor of endocytosis), and inhibition of uptake by chemical methods that prevent lipoprotein/LDLR interactions. In addition, CRIB cells (Flores and Donis, 1995; Flores et al., 1996) lack LDLR based on failure to bind anti-LDLR antibodies. The loss of infection by inhibition of endocytosis with anti-LDLR antibody indicates that LDLR-mediated endocytosis may be the main mechanism of virus entry (Agnello et al., 1999). However, the observation that there was a low background of infection associated with high levels of infecting virus indicated the presence of other, low-affinity receptor molecules.

The possibility of the CD46 molecule as a cell surface receptor for BVDV has been proposed (Rümenapf et al., 2000; Maurer et al., 2002). The CD46 protein is a known receptor for measles virus and herpesvirus 6 (Greenstone et al., 2002). Antibodies against the CD46 molecule, a complement regulatory protein, inhibited infection with BVDV. Expression of the bovine CD46 protein in porcine cells increased plaquing efficiency of cytopathic BVDV forty- to hundredfold. Expression of CD46 in nonpermissive cells did not confer susceptibility.

VIRAL FACTORS

The outer envelope of the BVDV virion contains the structural proteins E^{ms}, E1, and E2. These proteins are highly glycosylated and possess the major antigenic determinants of the virus. For a more complete discussion concerning the biology and structure of these proteins, refer to Chapter 3. Recent work has demonstrated that two of these proteins, E^{ms} and E2, play important roles in the attachment of the virus particle to the cell surface. Hulst and Moormann (1997), working with purified CSFV E^{ms} and E2 synthesized in insect cells, showed that E^{ms} added to susceptible cells prior to infection (100 µg/ml) could irreversibly bind to cells and prevent infection. Purified E2 at 10 µg/ml also provided 100% inhibition of infection but this inhibition was reversible and required additional supplementation of E2 to maintain inhibition. Following removal of E2, infection still occurred, presumably by virus particles bound to the surface of the cells. Treatment

with 100 µg/ml E^{ms} released these particles. The difference between concentration of proteins required to reach complete inhibition and differences in binding properties indicated that these proteins bound to different molecules on the cell surface. These differences also indicate that the E2 binding site is of lower prevalence.

Experiments have demonstrated that dengue virus (Hilgard and Stockert, 2000) and BVDV (Iqbal et al., 2000) E^{ms} molecules first bind to cell surface heparin sulfate proteoglycans and sulfated heparin-like glycosaminoglycans, respectively. This type of initial virus/cell interaction has been reported for a number of viruses that utilize two or more cell surface receptors (Mettenleiter et al., 1990; Okazaki et al., 1991; Jackson et al., 1996; Chen et al., 1997; Jusa et al., 1997; Krusat and Streckert, 1997; Asagoe et al., 1997). E^{ms} binds to both permissive and non-permissive cells because of the near ubiquitous presence of glycosaminoglycans (Iqbal et al., 2000). Soluble E^{ms} added to medium blocked replication of BVDV. As supporting evidence, E^{ms} binding was not observed when cells were treated with heparinase I or III, when soluble glycosaminoglycans were present, or to Chinese hamster ovary (CHO) cells that did not produce glycosaminoglycans or heparin sulfate. These lines of evidence led to the hypothesis that binding of E^{ms} to cell surface glycosaminoglycans is the initial event in viral attachment. Subsequently, Iqbal and McCauley (2002) showed that the conserved KKLENKSK motif near the C-terminus of the E^{ms} protein mediated binding of the glycosaminoglycan molecule.

The mechanism of release of genomic RNA into the cytoplasm of the cell is unclear but probably involves acidification of endocytic vesicles (Flores and Donis, 1995; Flores et al., 1996). Uptake of virus appears to occur by endocytosis and not by a membrane fusion mechanism. Treatment of BVDV-infected MDBK cells with phenylarsine oxide, an inhibitor of endocytosis and inhibitors of endosome acidification (chloroquine and ammonium chloride) prevented uptake of virus in the presence of PEG (Flores and Donis, 1995). Dengue virus, a member of the *Flaviviridae*, has been shown to traffic the major clathrin-dependent endocytic pathway during infection (Hilgard and Stockert, 2000). Work with West Nile virus demonstrated that acidification (<pH 6.6) caused rapid loss of viral infectivity (Gollins and Porterfield, 1986). Ammonium chloride also inhibited uncoating of virus and infection, demonstrating the dependence on acid pH in the infection process.

Taken together, these results suggest that attachment and entry of BVDV into a cell is a multistep process. First, the virus attaches to the cell surface through interaction of E^{ms} envelope protein and a docking glycosaminoglycan receptor molecule. This step can occur in both susceptible and nonsusceptible cells because these molecules are present on most cells. It appears that binding to the docking receptor is insufficient by itself to bring about uptake of the virus (Flores and Donis, 1995; Flores et al., 1996). Entry into the cell is further mediated by attachment of the E2 envelope protein to the LDLR receptor and internalization via endocytosis. This is probably the rate-limiting step due to the relatively low abundance of the LDLR on most cell types (Agnello et al., 1999). As was stated earlier, the presence of other, minor receptor molecules cannot be ruled out at this time.

INTERACTIONS WITH CELLULAR FACTORS DURING REPLICATION

Upon entry into the cell, the genomic RNA must act as mRNA, directing the translation of viral proteins. Viral proteins participate in the necessary functions for RNA replication, protein processing (protease cleavages), and protein trafficking, but are insufficient by themselves to bring about all of these events. Recruitment of cellular factors is necessary for the successful completion of the replication process. Many of these factors are known, but the identity and function of others remain elusive.

Probably the most important of the cellular functions utilized by BVDV for replication is protein synthesis. This process is much too complex to be encoded by a simple virus. Thus, BVDV must utilize the existing translational factors employed by the cell. In noninfected cells, eukaryotic cap-dependent translation initiation begins by the binding of eIF4E (the cap binding protein), as part of the heterotrimeric eIF4F complex (composed of eIF4A, eIF4E, and eIF4G), to the 5' m⁷G cap of the mRNA molecule (Figure 11.1A). The 40S small ribosome subunit, associated with the ternary complex eIF2/GTP/Met-tRNA_i, eIF2 and eIF3, forms the 43S preinitiation complex. The binding of the 43S subunit to the mRNA is directed by the eIF4F complex and results in the formation of the 48S complex. The ATP-dependent helicase activity of the eIF4A subunit is thought to unwind the RNA, allowing the binding of the 43S complex to the mRNA.

Both eIF1 and eIF1a are necessary for proper assembly of the 48S preinitiation complex as well as

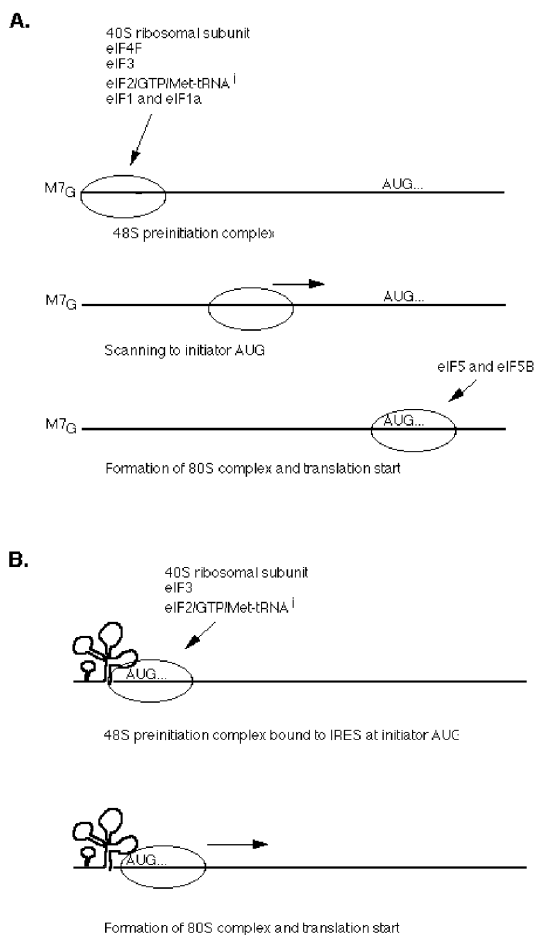


Figure 11.1. Translation initiation of cellular transcripts as compared to that of the pestivirus genomic RNA.

A. Eukaryotic translation initiation takes place by binding of the cap structure (M⁷G) as directed by the eIF4F complex. The attachment of the 40S small ribosomal subunit and other factors necessary to form the 48S preinitiation complex are illustrated. The scanning competent complex moves down the transcript until encountering the initiation codon, at which point eIF5 and eIF5B direct formation of the 80S ribosomal complex and initiation of translation. **B.** Formation of the 48S preinitiation complex at the BVDV IRES requires only the 40S small ribosomal subunit, eIF3 and eIF2/GTP/Met-tRNA_i. This results in the assembly of the translation-competent 80S ribosomal complex at the initiator AUG and translation of the BVDV polyprotein.

making the 48S preinitiation complex competent for scanning to the initiation codon. The scanning process to the initiation codon also requires eIF4B and eIF4F. At the initiation codon, eIF5, through interaction with both eIF2 and eIF3, causes the hydrolysis of the GTP moiety of eIF2, resulting in the

release of eIF2-GDP. Initiation factor eIF5B, again with hydrolysis of GTP, directs the attachment of the 60S ribosomal subunit in the formation of the translationally competent 80S ribosome. For a more detailed discussion of the eukaryotic protein translation initiation process, see Pestova et al. (1998) and Pestova et al. (2001).

POLYPROTEIN TRANSLATION

Following the uptake and the release of the viral genome, the next critical event in the replication cycle is the production of viral proteins, both those necessary for replication and those that interact with host factors to generate a cellular environment optimized for production of virus progeny. The BVDV genomic RNA functions as mRNA for the immediate synthesis of a large viral polyprotein that is encoded in the single open reading frame (ORF) of the virus. The ORF encodes a large protein of approximately 4000 amino acids that is co- and posttranslationally cleaved to produce the individual, mature proteins. The proteins, in order of placement within the genome from N- to C-terminus are NH₂-N^{pro}-capsid-E^{ms}-E1-E2-p7-NS2/3-NS4a-NS4b-NS5a-NS5b-COOH. The envelope glycoproteins—E^{ms}, E1, and E2—are translocated into the lumen of the endoplasmic reticulum for further posttranslational modification (Rümenapf et al., 1993). The E1 and E2 are inserted into the membrane of the endoplasmic reticulum during translocation while E^{ms} remains soluble.

A major difference between the BVDV genomic RNA and most cellular mRNA molecules is the presence of a long 5' nontranslated region (5' UTR) containing a high degree of secondary structure (Figure 11.2) as well as multiple upstream AUG codons. This roughly 390 base region, termed the internal ribosome entry site (IRES), functions to direct the attachment and assembly of the ribosome at the initiation codon of the ORF, insuring that initiation of translation begins at the proper AUG codon.

Computational analysis of the 5' untranslated region of pestivirus genomes revealed the presence of higher order folding and secondary structure (Brown et al., 1992; Deng and Brock, 1993; Le et al., 1995). The presence of multiple AUG codons was noted, several with stronger matches to the Kozak consensus sequence (Kozak, 1987) than the authentic initiation codon (Deng and Brock, 1993). These analyses resulted in the development of a model of secondary structure that revealed a series of stem-loops that were highly conserved structurally between BVDV and CSFV. These tertiary structures were conserved despite the sequence divergence between the two viruses. Brown et al. (1992) used double- and single-strand specific RNases to confirm the structure of the stem-loop structures and further found them to be highly similar to that described for the IRES of HCV (Tsukiyama-Kohara et al., 1992). Modeling studies by Le et al. (1995) predicted stem-loop structures as well as a pseudoknot in the BVDV and CSFV 5'

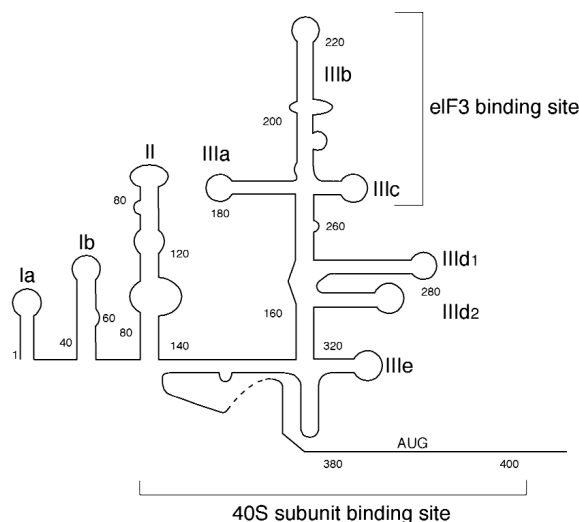


Figure 11.2. The secondary structure of the 5' UTR/IRES of the BVDV genomic RNA. The conserved stem-loop structures of this region are illustrated, along with the location of the binding sites of eIF3 and the 40S ribosomal subunits (Pestova et al., 2001). The nucleotide sequence is not shown because of variation between BVDV strains.

UTRs that were consistent with prior RNase sensitivity studies (Brown et al., 1992). The pestivirus IRES also contained a structural motif that was proposed to base pair with the 3' end of the 18S rRNA, in a manner similar to that described for the picornaviruses (Deng and Brock, 1993; Le et al., 1995). This base pairing would occur at sequences immediately 5' of the AUG initiation codon. It was proposed that this base pairing between the viral and 18S rRNA functioned in initiation of cap-independent translation by positioning of the ribosome.

The first description of the 5' UTR as a functional IRES was by Poole et al. (1995). In vitro transcription of the 5' UTR along with the p20 (N^{pro}) coding sequences of the BVDV ORF yielded expression of the p20 protein in an in vitro cell-free expression system. Inclusion of the 5' UTR as a spacer between the CAT and luciferase genes in a bicistronic construct yielded expression of both genes. Expression studies using constructs with both genes without a spacer resulted in only CAT expression. Deletion of nucleotides 173 through 236 of the 5' UTR reduced expression of luciferase by about two-thirds. These results suggested that the BVDV 5' UTR possesses IRES activity similar to that of HCV. Rijnbrand et al. (1997), in a separate study, demonstrated that the CSFV IRES sequences functioned similarly when used as a spacer in a bicistronic construct. Mutational analysis of the nucleotide sequences that formed the pseudoknot revealed that RNAs unable to form base pairs in stem II of the pseudoknot were translationally inactive. However, translational activity could be restored by introduction of compensatory base changes that restored the stem loop structure (Rijnbrand et al., 1997; Fletcher and Jackson, 2002). Neither an AUG codon 7 base upstream of the authentic initiation AUG codon nor an AUG downstream could initiate translation. This indicates that scanning by the ribosome on the IRES is limited to a very small region.

A genetic approach to dissection of BVDV IRES function (Chon et al., 1998) using mutational analysis through a number of in vitro-introduced deletions or insertions, showed that stem loops Ia and Ib were dispensable for efficient translation, and IIIb and IIIe were partially required (Figure 11.3). Deletions or insertions in II, IIIa, IIIc or IIId could cause a tenfold or greater reduction in translation. Confirmation of these data was provided by chemical and enzymatic footprinting (Sizova et al., 1998), which demonstrated that the translation initiation factor eIF3 bound to and protected distinct regions of domain III, particularly IIIb and IIIc (refer to

Figure 11.2). Further, deletions of these sequences abrogated translation. They suggested that eIF3 bound the IRES in a sequence- or structure-specific manner. In UV cross-linking experiments to determine which subunits of the 10 protein subunit eIF3 complex actually contacts the IRES, 4 subunits were found to be labeled by the radiolabeled RNA (p170, p116, p66, and p47). The p116 and p66 subunits both contain RNA recognition motifs and thus are likely to be determinants of the interaction of eIF3 with the IRES (Sizova et al., 1998).

Pestova and Hellen (1999) provided evidence that the IRES was bound independently of eIF3 and the 40S ribosomal subunit. The IRES contains complex structure determinants (refer to Figure 11.2) that mediate attachment of the 48S ribosomal subunit complexes to the initiation codon (Pestova et al., 1998). Determinants of domain III mediate eIF3 binding. Pestivirus translation initiation is dependent on eIF3, eIF2, GTP, and Met-tRNA_{met} (48S subunit assembly) but not on any constituent of the eIF4G subunit (refer to Figure 11.1B). eIF3 enhances 48S complex formation, probably by stabilizing ribosomal complexes by its interaction with the IRES, and is absolutely required for subsequent subunit joining to form active 80S ribosomes (Sizova et al., 1998). The probable function of eIF3 is to recruit factors eIF1 and eIF5 to the 48S complex (Pestova and Hellen, 1999). Using cryoelectron microscopy, Spahn et al. (2001) demonstrated that binding of the HCV IRES caused a conformational change in the 40S ribosomal subunit that resulted in closing of the mRNA binding cleft, and in IRES-mediated positioning of the initiation codon in the P site of the ribosome. This was the first description of a viral RNA that actively manipulates the structure of the transcriptional machinery, resulting in the promotion of translation initiation without the assistance of otherwise necessary initiation factors (Spahn et al., 2001).

Several groups have reported that initiation factors eIF4A, eIF4B, and eIF4F were not required for translation initiation from the pestivirus IRES (Rijnbrand et al., 1997; Chon et al., 1998; Pestova and Hellen, 1999; Sizova et al., 1998; Fletcher et al., 2002). These findings were derived from different types of experiments, including reconstituted in vitro translation initiation systems (Pestova and Hellen, 1999) and coexpression of picornavirus 2A protease (Rijnbrand et al., 1997; Chon et al., 1998). In addition, this IRES-driven translation was shown to be independent of the translation initiation factor eIF-4F by coexpression of the poliovirus 2A protease

that cleaves eIF4F. This protease-inhibited cap-dependent translation in picornavirus-infected cells (Rijnbrand et al., 1997; Chon et al., 1998; Pestova et al., 1998; Pestova et al., 2001; Fletcher et al., 2002). The report by Pestova et al. (1998) was the first describing the lack of reliance by the pestivirus IRES on canonical translation initiation factors that are necessary for normal cellular cap-dependent translation initiation. It was concluded that pestivirus protein translation initiation is simpler than that employed by the picornaviruses and more closely resembles prokaryotic translation initiation. Translation initiation from the flavivirus or pestivirus IRES requires so few translation initiation factors that a simpler, equally efficient means of translation initiation is unlikely (Pestova et al., 1998). Cellular RNAs that contain an IRES are generally those that are expressed under stress conditions (i.e., heat shock, reactive oxygen species) when the activities of eIF4B, E, and F are reduced (Pestova et al., 1998).

The 5' end of the IRES was mapped to between nucleotides 28 and 75 (Rijnbrand et al., 1997; Chon et al., 1998). The 3' end of IRES extends into Npro coding sequences of the ORF (Chon et al., 1998) as far as 51 bases (Fletcher et al., 2002) and possesses single-strandedness that appears important for ribosome binding and translation initiation. Studies mapping the termini of the IRES were done utilizing *in vitro* systems where the IRES was directly fused to the ORF encoding the expression reporter. Additional work showed that efficient IRES function, in the context of translation from a functional replicon, required the 5' Ia stem-loop structure (Yu et al., 2000). Utilizing the subgenomic RNA replicon DI9c (Behrens et al., 1998), translation was shown to be inefficient when the Ia stem-loop structure was mutated or missing. In addition, it was found that the Ia structure was important in RNA replication as well. The authors postulated that this structural motif may be involved in RNA:RNA interactions that may be necessary for committing the RNA molecule to function as template for either translation or RNA replication.

PROCESSING OF ENVELOPE GLYCOPROTEINS BY CELLULAR ENZYMES

The BVDV polyprotein, encoded by the single ORF of the genomic RNA, is co- and posttranslationally processed by both host and viral proteases to yield the mature viral proteins. The protease cleavages of the nonstructural proteins are carried out by the NS2/3 serine protease (Wiskerchen and Collett, 1991; Tautz et al., 1997; Xu et al., 1997). However,

the processing of the virus structural proteins, encoded in the N-terminal one-third of the polyprotein, is carried out by host signal peptidases (Figure 11.3A) following translocation into the lumen of the endoplasmic reticulum (Figure 11.3; Rümenapf et al., 1993; Lin et al., 1994). A model of this translocation and proteolytic processing has been proposed (Rümenapf et al., 1993). Cotranslationally, the envelope proteins are translocated into the ER utilizing a signal sequence located between the capsid and E^{ms} proteins. In doing so, the ribosome attaches to the Sec61p complex, a protein translocation channel in the ER membrane. The signal peptide is inserted into the membrane and the nascent protein chain is translocated into the lumen of the ER in a loop structure (Matlack et al., 1998). A rapid signalase cleavage occurs at the N-terminus of the E^{ms} protein, possibly leaving the capsid protein attached to the cytoplasmic side of the membrane.

The core (capsid) protein of HCV is released from the endoplasmic reticulum membrane by the cellular protease SPP (McLauchlan et al., 2002), and it is likely that process occurs in a similar manner in BVDV-infected cells. Transfer of the remaining E^{ms}/E1/E2 envelope protein precursor continues with interruption by two hydrophobic domains at the end of the E1 protein. The first hydrophobic domain acts as a stop transfer for the nascent protein, and the second acts as a signal sequence to begin translocation of the E2 protein. Both may function to anchor the E1 protein into the membrane. There are similar stop/start transfer signals at the C-terminus of the E2 region. The internal signal sequences of the E^{ms}/E1/E2 precursor are the only determinants for compartmentalization of the mature envelope proteins (Wu, 2001). Cleavage of the E^{ms}/E1/E2 precursor from the downstream translation product occurs rapidly. Processing of the precursor begins with the signalase cleavage at the E1/E2 border. This is followed by the separation of the E^{ms}/E1 proteins and is probably dependent on a conformational change taking place following the initial E2 cleavage reaction. The E^{ms} protein forms homodimers by intermolecular disulfide bond formation and, having no hydrophobic transmembrane domain, remains soluble and associates with the E1-E2 heterodimers by an unknown mechanism (Hulst and Moorman, 2001). Unassociated E^{ms} is also secreted into the extracellular domain and can be found free in the medium of infected cells cultured *in vitro* (Rümenapf et al., 1993). As discussed previously, E^{ms} is essential for virus attachment and uptake (Hulst and Moorman, 1997).

A.



B.

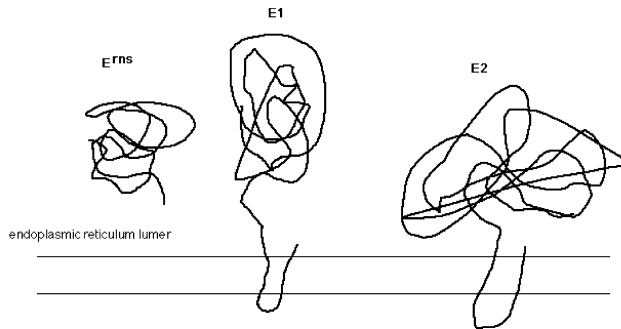


Figure 11.3. Proposed mechanism for transporting and processing of pestivirus envelope proteins. **A.** Genomic organization of the BVDV genome showing cleavage sites of the mature proteins with those cleaved by host signalases denoted by asterisks. The sizes of the proteins are not drawn to scale. **B.** Import into the lumen of the endoplasmic reticulum and cleavage of the mature proteins from the polyproteins result in the release of free E^{ns} into the lumen of the endoplasmic reticulum, while cleavage of the E1 and E2 proteins results in retention of the membrane anchors in the membrane, probably facilitating downstream processing, heterodimerization and transport.

The E1 and E2 proteins form disulfide-linked heterodimers after proper folding of the proteins in association with the endoplasmic reticulum protein calnexin (Branza-Nichita et al., 2001). The folding of these proteins is dependent on intramolecular disulfide bridge formation and varies in the rate this occurs between the two envelope proteins. The folding of the E2 protein is rapid, showing completion within 2.5 minutes; the folding of the E1 proteins takes approximately 30 minutes. Thus the rate of folding of the E1 protein is the rate-limiting step in the formation of E1-E2 dimers. The interaction of envelope proteins with the lectin-like calnexin molecule is dependent on the removal of glucose moieties on the N-linked glycans by endoplasmic reticulum α -glucosidases (Branza-Nichita et al., 2001). If the removal of the glucose moieties does not occur, interaction with calnexin is inhibited and the improperly folded proteins are targeted for degradation by the proteasome (Branza-Nichita et al., 2002). Decrease in size of cleaved proteins that indicated trimming of sugar moieties on the side-chains has been reported (Rümenapf et al., 1993). Jordan et al. (2002a) demonstrated that inhibition of α -glucosidase removal of terminal glucose moieties

from the nascent E2 glycoprotein prevented golgi processing of the E2 glycoprotein with the loss of production of infectious progeny virus.

RNA REPLICATION

Replication of the BVDV genomic RNA occurs downstream of polyprotein translation, being dependent on the presence of viral proteins that function specifically in RNA replication. Replication of the genomic RNA involves the copying of the infecting plus-sense RNA into minus-sense RNA copies, forming the replicative form (RF). The negative-strand of the RF is copied by strand-displacement, giving rise to the RNA replicative intermediate (RI), and releasing the displaced plus-sense RNA strand when nascent strand transcription is complete. The RF is reutilized, primarily by recycling of the negative-sense. The kinetics of synthesis showed that the rate of synthesis of the positive-sense strand at 12 hours postinfection was 10 times that of the negative strand (Gong et al., 1996). At any given time, there were approximately 6–7 nascent plus-stranded RNA strands on the template RI (Gong et al., 1998).

Viral proteins known to participate in RNA repli-

cation include NS2/3, NS4B, NS5A, and NS5B. NS2/3 possesses RNA helicase and nucleotide triphosphatase activities essential for RNA replication (Warrener and Collett, 1995; Grassmann et al., 1999; Gu et al., 2000). NS5B contains the GDD amino acid motif characteristic of RNA-dependent RNA polymerases and has been demonstrated to possess RNA polymerase activity and be essential for RNA replication (Zhong et al., 1998; Lai et al., 1999). NS4B is an integral endoplasmic reticulum protein that, besides being part of the multiprotein complex composed of NS3, NS4B, and NS5A, can also play a role in suppression of the cytopathic phenotype by mutation of the highly conserved tyrosine residue at position 2441 (Qu et al., 2001). This apparent tripartite protein complex may be associated with the endoplasmic reticulum membrane via the transmembrane domain of NS4B (Qu et al., 2001). The function of NS5A remains elusive and is discussed later with its known interactions with cellular proteins.

Currently, little is known concerning the identity and the role of host factors in the replication of the BVDV genomic RNA. Highly conserved RNA secondary structures at both the 5' and 3' ends of the BVDV genomic RNA indicate functional roles (Becher et al., 2000; Yu et al., 2000). As illustrated by the function of the IRES, these secondary structures probably bind specific cellular factors, as well as viral proteins, that participate in RNA replication (Deng and Brock, 1993; Becher et al., 2000). The requirement for the secondary structure contained in the 5' nontranslated region of the BVDV genomic RNA in viral replication has been analyzed (Yu et al., 2000). Stem-loop Ia, found outside the IRES sequences at the extreme 5' end of the genomic RNA (Rijnbrand et al., 1997; Chon et al., 1998), appears to regulate the switching of the genomic RNA of BVDV as template for either translation or RNA replication (Behrens et al., 1998; Yu et al., 2000; Li and McNally, 2001). This was supported by findings that regulation of RNA replication and translation was outside the IRES region and was probably due to interaction with regulatory proteins or viral protein(s) associated with specific host protein translation complexes (Li and McNally, 2001).

Several reports have demonstrated interactions of cellular proteins with 3' nontranslated region secondary structures (both plus and minus strands) of flaviviruses. Several of these proteins have been identified (Blackwell and Brinton, 1995; Blackwell and Brinton, 1997; Ito and Lai, 1997; Li et al., 2002). Two separate reports may provide evidence

for one possible function of NS5A. The first study demonstrated that the cellular protein elongation factor-1 α (EF-1 α) recognized and bound to highly conserved secondary structure found on the plus-strand of the 3' nontranslated region of West Nile virus (Blackwell and Brinton, 1997). The second demonstrated that NS5A protein of BVDV interacts specifically with EF-1 α (Johnson et al., 2001). Taken together, these data indicate that binding of EF-1 α to both the secondary structure of the 3' nontranslated region and to NS5A may act to bring the genomic RNA template and the NS5A containing endoplasmic reticulum membrane-bound replication complex into the correct position or orientation for RNA replication to take place. EF1- α plays numerous roles in RNA sorting and regulation of expression of mRNA in eukaryotic cells and may play a role in RNA replication of BVDV.

The RNA-binding proteins TIA-1 and TIAR have been identified as cellular proteins that interact with the 3' nontranslated region of West Nile virus (Li et al., 2002). In contrast to EF-1 α , TIAR and TIA-1 bind to the 3' nontranslated region of the minus strand of the West Nile virus genomic RNA. West Nile virus grew to significantly lower titers in murine TIAR^{-/-} knockout cells; however, growth was not eliminated. TIA-1^{-/-} knockout cells showed no decrease in final virus titer but these levels were reached 6 hours later than those grown in normal control cells, indicating distinct requirements for the two proteins. The identification of proteins that differentially bind to the plus and minus strands of the genomic RNA may indicate a mechanism to distinguish between the two strands during replication. These data demonstrate that host proteins that normally interact with cellular RNA are essential to the flavivirus RNA replication process. It is expected to be similar in BVDV RNA replication.

The function of some BVDV proteins may be regulated through phosphorylation by cellular protein kinases. The NS5A protein of BVDV was shown to be phosphorylated (Reed et al., 1998). The ability of NS5A to interact with both cellular and viral proteins and perhaps regulate their function may be dependent on its phosphorylation state (Kapoor et al., 1995; Reed et al., 1998). The phosphorylation of the BVDV NS5A protein indicates that it is associated with serine/threonine protein kinases (Reed et al., 1998). Kinase inhibitor studies indicated that the associated kinases belong to the CMGC family of protein kinases (Reed et al., 1997). This interaction has been postulated to regulate viral replication and possibly host gene expression, whether through trans-

port of NS5A into the nucleus or through interactions with cellular signaling pathways. An example of the latter includes the interaction with the host double-stranded RNA-activated protein kinase (PKR) by the HCV NS5A protein. This interaction inhibits the host interferon antiviral response, including inhibition of the phosphorylation of translation initiation factor eIF-2 α and subsequent decrease in protein translation (Gale et al., 1997).

CELL DEATH

Apoptosis, or programmed cell death, is the genetically and biochemically defined mechanism of cellular suicide that may be induced for a number of physiological reasons. From a virological standpoint, this is one of the first lines of defense against viral infection. Its primary purpose is to kill the virus-infected cell before the virus replicates and spreads to neighboring cells. Apoptosis is also a means to kill the cell without inducing an inflammatory response that may damage surrounding tissue (Steller, 1995). Apoptosis is characterized by many cellular changes, including cellular swelling, loss of plasma membrane integrity, loss of mitochondrial membrane potential, release of compartmentalized molecules into the cytoplasm, proteolytic cleavage of proteins (both activation and inactivation of function), and degradation of nuclear DNA. Apoptosis is induced in cells by two major pathways, the extrinsic and intrinsic pathways. The extrinsic pathway is induced when an extracellular signal is received and transduced into the cytoplasm. This results in the activation of caspase 8, initiating the caspase-mediated destruction of the cell. The intrinsic pathway is activated by an intracellular stimulus that results in loss of mitochondrial membrane potential ($\Delta\psi_m$) and release of cytochrome c. Cytochrome c, free in the cytoplasm, is bound by APAF-1 and, along with ATP, activates caspase 9, thus beginning the caspase-mediated cellular destruction.

Many viruses encode proteins that interact with cellular defense mechanisms, resulting in the inhibition of apoptosis until viral replication steps have been conducted, allowing production of maximal levels of progeny virus. At this point, no inhibitor of apoptosis has been specifically identified in the BVDV genome. The hallmark of the cp BVDV strains is the induction of cell death in cultured epithelial cells soon after infection, generally within 24–48 hours. The inability of the cp BVDV strains to infect susceptible cells without inducing cell death is considered loss of function, because the more prevalent noncytopathic BVDV (ncp BVDV)

strains possess this ability. The loss of the ability to prevent cell death by cp BVDV strains appears to be related to the genetic changes that occur within the NS2/3 protein coding sequences that give rise to the formation of NS3 (Hoff and Donis, 1997; Lambot et al., 1998), although other mechanisms cannot be ruled out (Bruschke et al., 1997; Vassilev and Donis, 2000; Qu et al., 2001).

Late in the infection process, cells infected with cp BVDV strains show many of the classic signs of apoptosis. These include rounding of cells, cleavage of nuclear DNA to oligonucleosomal fragments, and cleavage and inactivation of poly (ADP-ribose) polymerase, a nuclear enzyme important in DNA repair (Zhang et al., 1996; Hoff and Donis, 1997). In an investigation of the mechanism of induction of apoptosis in cp BVDV-infected cells, Grummer et al. (2002a) demonstrated that cp BVDV-infected cells induced the intrinsic apoptotic pathway. This was shown by translocation of cytochrome c into the cytoplasm, increased expression of APAF-1, and increased caspase 9 activity that is indicative of APAF-1 activation. Inhibition of loss of mitochondrial $\Delta\psi_m$ and subsequent loss of cytochrome c into the cytoplasm delayed onset of apoptosis. Additionally, treatment of cells with apoptosis inhibitors delayed induction of apoptosis (Grummer et al., 2002b). Disruption of the $\Delta\psi_m$ disrupts normal cellular oxidation/reduction. Schweizer and Peterhans (1999) provided evidence that cp BVDV-infected cells show an increase in reactive oxygen species, indicative of oxidative stress that preceded caspase activation. Antioxidants that protected the cell from oxidative stress, prevented apoptosis. Interestingly, this had no effect on virus replication and virus titers, indicating that the loss of $\Delta\psi_m$ and induced oxidative stress plays no role in cp BVDV replication (Schwiezer and Peterhans, 1999; Grummer et al., 2002a).

The envelope glycoprotein E^{ms} has been reported to possess RNase activity (Schneider et al., 1993; Hulst et al., 1994), the function of which is unknown. The effect of E^{ms} on cells infected with CSFV was investigated by Bruschke et al. (1997). This was done because of the immunomodulatory effects of RNases (Tamburrini et al., 1990; D'Alessio, 1993) and the known immunosuppression associated with pestiviral infections. Treatment of lymphocytes from porcine, bovine, ovine, and human sources with E^{ms} completely inhibited concanavalin A activation in vitro. In addition, protein synthesis was inhibited in the E^{ms}-treated cells without disruption of the plasma membrane. Examina-

tion of cells following treatment revealed that apoptosis was induced in the treated cells. These results suggest that E^{ms} may, at least in part, be responsible for the leukopenia observed in pestivirus infected animals. Earlier work had shown that E^{ms} was secreted in the medium of cultured cells (Rümenapf et al., 1993). If this is true in vivo, circulating 0.0 may be able to effect cell death in lymphocytes without direct viral infection and replication. The mode of entry into the susceptible cell remains a mystery. Langedijk (2002) demonstrated that short peptides corresponding to the C-terminus of E^{ms} were transported across the plasma membrane and targeted to the nucleolus. The region involved, as short as 13 amino acid residues in length, appeared to be associated with conserved basic residues. These C-terminal sequences, when attached to unrelated proteins, would still function to mediate transport into the cell. The translocation was rapid, occurring in 1 minute or less, and was not dependent on energy or cell surface receptors. Delivery of the peptide to the nucleolus may indicate that E^{ms} has a role in gene expression or protein synthesis modulation.

BVDV, like all members of the Flaviviridae, utilize the endoplasmic reticulum in their replication process with the envelope proteins being inserted into the membrane of the endoplasmic reticulum for further modification. When the endoplasmic reticulum experiences loss of Ca^{2+} or the accumulation of misfolded or unassembled proteins, the endoplasmic reticulum stress response is triggered. This acts to slow translation of proteins and is mediated through phosphorylation of eIF-2 α by the ER kinase PERK (Ma et al., 2002). This is maintained until the problem of the accumulated proteins is resolved (Kaufman, 1999). PERK phosphorylation of eIF-2 α also leads to transcriptional induction of endoplasmic reticulum chaperone proteins as well as transcriptional repression of bcl-2 (anti-apoptotic protein). If the PERK-mediated protein translation interruption is of sufficient duration, apoptosis may be induced by decreased levels of bcl-2 (Friedman, 1996). Jordan et al. (2002b) demonstrated that MDBK cells infected with cp BVDV induce the ER stress response. This response was characterized by activation of PERK, hyperphosphorylation of eIF-2 α , and decreased transcription of bcl-2. In addition, increased caspase 12 activity and decreased glutathione levels were noted. The latter would be a consequence of decreased bcl-2 protein levels and could potentially contribute to increased susceptibility to reactive oxygen species (Schweizer and Peterhans, 1999; McCullough et al., 2000). Overex-

pression of bcl-2 in cells infected with dengue virus and Japanese encephalitis virus was demonstrated to change the cellular response to the infection from apoptotic to chronic (Su et al., 2001; Su et al., 2002).

INHIBITION OF SIGNALING

CYTOKINES

The innate immune response represents the first line of defense against an invading virus and occurs at the cellular level. Interferon production and induction of apoptosis represent two mechanisms a cell may employ to attempt to limit the infection. Synthesis and release of interferon act not only at the infected cell but also to notify other cells of the danger posed by the virus. Many viruses encode proteins that target components of the innate immune system to limit the response, giving the virus time to replicate to maximal levels. BVDV appears to be no exception; however, the mechanisms utilized to bring this about remain unclear. Adler et al. (1994), working in vitro with bovine bone marrow-derived macrophages (BBMM), demonstrated that only ncp BVDV strains primed BBMM for enhanced nitric oxide production in the presence of Salmonella. Infection of cells by cp BVDV resulted in cytopathic effect (apoptosis), demonstrating that the two biotypes acted differently in affecting specific functions of the host. In a later study, Adler et al. (1996), again working with BBMM, showed that infection with both biotypes resulted in decreased production of tumor necrosis factor α (TNF α) upon stimulation with Salmonella, while other specific macrophage functions were not affected. This suggested that decline in the ability to produce TNF α may contribute to immunosuppression often observed in BVDV infections. In addition, the infected BBMM also produced a substance that primed uninfected cells for decreased nitric oxide production and for apoptosis following treatment with lipopolysaccharide (Adler et al., 1997; Jungi et al., 1999). This substance was believed to be interferon, based on its physicochemical properties (Adler et al., 1997; Perler et al., 2000).

INTRACELLULAR SIGNALING INHIBITION

The production of interferons by virus-infected cells is an important function of the innate immune response. The mechanism of inhibition of interferon synthesis by ncp BVDV in infected cells remains unclear. ncp BVDV strains, but not cp BVDV strains, possess a function that inhibits interferon production in response to infection or treatment with

dsRNA, but not the response to exogenous interferons (Schweizer and Peterhans, 2001). dsRNA is a molecule produced during the infection process by most viruses and has been shown conclusively to be a specific trigger of apoptosis and interferon production (Jacobs and Langland, 1996; Kibler et al., 1997). This inhibition was observed only in cells that had been infected with the ncp BVDV strain for at least 12 hours, indicating that inhibition was virus-induced. ncp BVDV inhibited induction of apoptosis by dsRNA but not by staurosporine or actinomycin D. In addition, this inhibition was through an unknown intracellular mechanism and not by inhibition of uptake of the dsRNA. This suggested that some component encoded by ncp BVDV interfered specifically with intracellular signaling. By using different cell lines and different strains of ncp BVDV, Schweizer and Peterhans (2001) demonstrated that interference with apoptosis and interferon synthesis was a general and important function of ncp strains.

Baigent et al. (2002) extended the above studies to further characterize the lack of intracellular response in cells infected with ncp BVDV. As described by Schweitzer and Peterhans (2001), prior infection with ncp BVDV did not block the response to exogenous interferons but could block interferon and MxA gene transcription in response to dsRNA or infection by the heterologous virus Semliki Forest virus (SFV). Prior infection with ncp BVDV did not block induction of apoptosis by cp BVDV or SFV. The nature of the interferon response requires transcriptional up-regulation or posttranslational modification of specific transcription factors, including NF- κ B, ATF2, and c-jun. Examination of these proteins revealed that NF- κ B was not activated, and ncp BVDV infection did not block activation of NF- κ B by SFV or by tumor necrosis factor- α . The stress-activated kinases JNK1 and JNK2 were not activated nor were the transcription factors ATF2 and c-Jun phosphorylated. These events were not inhibited following superinfection by SFV following ncp BVDV-infection. Interferon regulatory factor-3 (IRF-3), a transcriptional activator responsible for the increased transcription of interferon genes, was translocated to the nucleus in ncp BVDV-infected cells but was shown to lack DNA binding activity in nuclear extracts (Baigent, et al., 2002). In addition, IRF-3 DNA binding activity was present in SFV-infected cells but could be blocked by prior infection with ncp BVDV.

HCV was recently shown to also inhibit IRF-3 transcriptional activation of interferon genes (Foy et

al., 2003) but by an apparently mechanistically different means. The HCV NS3/4A serine protease prevented the phosphorylation of IRF-3, blocking its translocation to the nucleus. This is in contrast to the IRF-3 in ncp BVDV-infected cells where IRF-3 is translocated to the nucleus but does not bind DNA. The two viruses appear to utilize two different mechanisms to achieve the same goal.

A recent report by Ruggli, et al. (2003) suggested that the pestivirus protein N^{pro} may play a role in interference with the induction of the interferon response. Porcine SK-6 cells infected with wild-type CSFV possessed the ability to resist induction of apoptosis when treated with dsRNA, and macrophages were inhibited from producing an α/β interferon response. When infected with CSFV mutants lacking the N^{pro} coding sequences, SK-6 cells were not protected from dsRNA-induced apoptosis and macrophages produced a type I interferon response in the absence of additional stimuli. In addition, reduced replication was noted for the N^{pro}-deficient mutant. These data imply that N^{pro} plays a role in the inhibition of the interferon response, the mechanism of which still remains to be determined.

CELLULAR REMODELING

Recent advances in functional genomics have made it possible to examine changes in gene expression in cells under a variety of conditions that allow an understanding of the basic mechanism(s) that bring about distinct cellular changes. These changes, indicated by increased or decreased transcription of specific genes, allow the dissection of the host response based on the function of the genes with altered expression levels. One functional genomics technology, serial analysis of gene expression (SAGE), was applied to examining gene expression changes that take place in BVDV-infected cells in response to the viral infection (Neill and Ridpath, 2003a, b). This analysis revealed that a number of gene expression changes occur following BVDV infection and that some of the changes act to increase efficiency of functions that are beneficial to viral replication, maturation, and release. Although expression level of genes involved in energy production and metabolism were relatively unchanged, genes encoding proteins involved in protein translation were altered in a pro-virus manner. Protein translation plays such an integral role in the replication of a virus that alterations in the translational machinery that enhances viral protein synthesis are advantageous. These changes included ribosomal proteins, elongation factors, and tRNA synthetases. In addition,

transcription of genes involved in transport of nascent polypeptides into the lumen of the endoplasmic reticulum was increased, indicating possible increased capacity for ER translocation and processing. One protein in particular, TRAM, functions in regulating the Sec61p pore complex through which the nascent polypeptide passes into the lumen of the endoplasmic reticulum (Hegde et al., 1998a). TRAM also regulates exposure of the nascent protein to the cytoplasm in response to pause transfer signals (Hegde et al., 1998b). This may be important for BVDV replication in allowing the correct processing of the structural proteins. Gene expression changes were also noted with unknown effect or benefit to the replicating virus. Several tubulin isoforms showed sharp declines in transcript numbers, indicating possible disruption of microtubule function. This may suggest a possible mechanism for the disruption of platelet production by BVDV-infected megakaryocytes. Proper microtubule function is essential for pro-platelet release, and disruption of the microtubule network would have a detrimental effect on this process.

VIRUS RELEASE

Little is currently known concerning release of infectious BVDV virions from the infected cell. This is one of the more confusing aspects of flavivirus/pestivirus biology because of apparent differences in the mechanisms of viral egress used by different viruses. Egress by budding from the cell surface was reported for West Nile (Sarafend) virus and was demonstrated by both transmission and scanning electron microscopy (Ng et al., 1994). Examination of release of virus in polarized epithelial cells by Chu and Ng (2002) showed that West Nile (Sarafend) was released from the apical surface, and Kunjin virus was released from both apical and basolateral surfaces. In addition, microtubules were shown to play a role in the sorting and transport of viral proteins to the cell surface by West Nile (Sarafend) virus, and disruption of the microtubule network had no effect on Kunjin virus maturation and egress (MacKenzie and Westaway, 2001; Chu and Ng, 2002). Transport of Kunjin virus to the cell surface from the golgi apparatus appears to be by movement of virus-containing vesicles and fusion with the plasma membrane.

The release of mature pestivirus particles appears at this point to more closely resemble that of Kunjin virus. A study examining location of viral proteins in cells infected with CSFV (Weiland et al., 1999) showed that the viral proteins were associated only

with released viral particles. Lack of detection of viral proteins in the plasma membrane of BVDV-infected cells was confirmed by Grummer et al. (2001) using indirect fluorescence microscopy, confocal microscopy, or FACS analysis. Using subcellular fractionation techniques, the envelope glycoproteins E^{rns} and E2 were found associated exclusively with intracellular membranes. In addition, with the possible disruption of microtubule networks in BVDV-infected cells (Neill and Ridpath, 2003), the microtubule-associated sorting of viral proteins required for surface budding would be affected. This provides further evidence for the use of an egress mechanism more closely resembling that of Kunjin virus.

BVDV REPLICATION CYCLE OVERVIEW

The mechanisms utilized by BVDV to enter a susceptible cell and replicate itself are becoming clearer. It is now possible to put together the picture of what takes place from attachment of the infecting virus particle to release of infectious progeny. As detailed in Figure 11.4, the infection process begins by attachment of the BVDV particle to the plasma membrane, probably first by attachment of E^{rns} to a docking surface glycosaminoglycan(s) followed by binding of the LDLR E2 (Figure 11.4A). The internalization of the virion is probably mediated by the LDLR receptor through endocytosis (Figure 11.4B,C), and release of the genomic RNA occurs following acidification of the endosomal vesicle (Figure 11.4D,E). The genomic RNA, now in the cytoplasm of the cell first acts as mRNA for translation of the polyprotein at least initially taking place on the endoplasmic reticulum (Figure 11.4F). At some point, a switch occurs that causes the genomic RNA to be used as template for RNA replication rather than for protein translation (Figure 11.4G). It is presumed that some of the newly synthesized daughter RNAs are then used for protein translation while others participate in RNA replication. The genomic RNAs also interact with the capsid protein followed by recognition of the cytoplasmic domains of the envelope proteins and budding into the lumen of the endoplasmic reticulum (Figure 11.4H,I,J). The passage of the immature particles through the endoplasmic reticulum and the golgi body result in the maturation of the particles by processing and glycosylation of the envelope proteins (Figure 11.4K). The virus-laden vesicles (Figure 11.4L) move through the cytoplasm to the cell surface (Figure 11.4M) where the vesicle fuses with the plasma

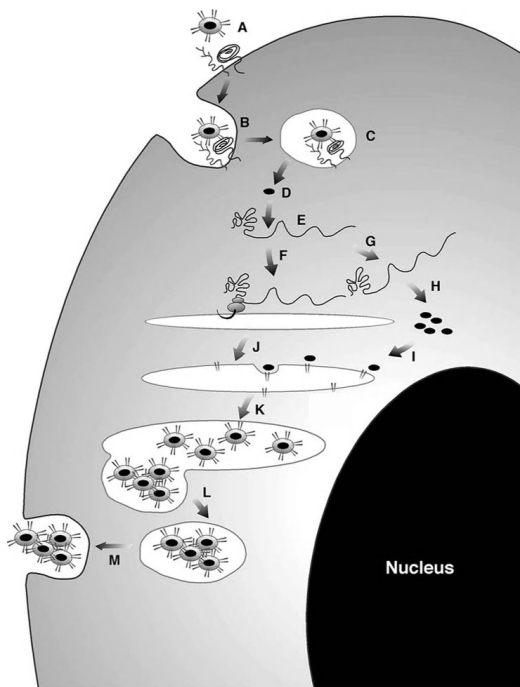


Figure 11.4. Overall view of BVDV infection, replication, and release of progeny virus derived using currently available information. Please see text for additional details.

membrane, releasing the virus particle into the extracellular environment. The budding of the capsid-bound genomic RNA and the immature virus particle into the lumen of the endoplasmic reticulum, followed by the release of mature particles directly from a vesicle fused with the plasma membrane, would explain why viral antigens are not found associated with plasma membrane of the infected cell.

FINAL REMARKS

Replication of an RNA virus in a susceptible cell is a complicated affair, requiring essentially all of the cell's resources. This is an intricate combination of cellular machinery and virus-encoded proteins working in concert, most often to the detriment of the cell, to bring about production of progeny virus. Some of these effects are obvious, but others are very subtle in their effect. Increasingly more experimental evidence is available showing the dependence on host cell proteins in carrying out many aspects of virus replication. BVDV is no different, although much of what we know is supported or inferred from work with other pestiviruses or flaviviruses. Regardless, much is left to do. This is par-

ticularly true in two areas. The first is how the virus interacts with the host cell in establishing a chronic infection in utero that results in the birth of a persistently infected calf. The second is the subtle interactions with the host immune system and how it is manipulated to delay both the innate and acquired immune responses. Continued work will give greater insight into replication strategy of pestiviruses and possible means to intervene, yielding better vaccination strategies and perhaps pharmacological treatments to prevent, inhibit, or moderate infection.

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12

Diagnosis

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INTRODUCTION

Infections with bovine viral diarrhea virus (BVDV) are endemic in many countries, leading to heavy economic losses for the cattle industry. Sweden was one of the first countries to introduce a national BVDV control program in 1993, which now forms the basis for control programs in many other countries (Moennig and Greiser-Wilke, 2003). The primary aim of BVDV control programs in Scandinavian countries is the identification of BVDV-free herds and prevention of reinfection of these herds so that there is a gradual decrease in the number of infected herds. A crucial requirement for the success of these programs is the availability of rapid, economical, and simple diagnostic methods that are highly sensitive and specific. In the Scandinavian model, different diagnostic tests are used for the detection of infection at various levels. Initially, bulk tank milk is screened for BVDV or anti-BVDV antibody using a reverse transcription-polymerase chain assay (RT-PCR) or an enzyme-linked immunosorbent assay (ELISA), respectively. If positive, identification of individual, persistently infected (PI) animals is undertaken. For this purpose, an ELISA for the demonstration of virus in blood has proved reliable (Bitsch and Ronsholt, 1995).

The Scandinavian model may work well in countries where cattle density is low and vaccination is not allowed. It may not work, however, in countries where both virus prevalence and cattle densities are high and where vaccination is permitted. Economic losses in these countries can be minimized by lowering the infection pressure (Moennig and Greiser-Wilke, 2003). No matter what control program is used, the identification and elimination of PI animals is of the utmost importance (Schelp and Greiser-Wilke, 2003), which is possible only if the diagnostic tests are highly efficient and reliable.

The diagnosis of BVDV infection can sometimes be made on the basis of history and clinical signs. However, clinical signs following BVDV infection are highly variable depending on viral strain, age, and immune status of the animal; reproductive status of the animal; and the presence of other pathogens. Thus, BVDV infection may result in subclinical acute infections; severe acute infections characterized by fever, leukopenia, and thrombocytopenia; persistent infections; reproductive disease presenting as congenital defects, repeat breeding, abortion, or mummification; enteric disease; respiratory disease; and immunosuppression. Because so many different types of clinical presentation are associated with BVDV infection, a diagnosis on the basis of history, clinical signs, and postmortem examination of dead animals can only be considered presumptive. Accurate and definitive detection of BVDV infection depends on laboratory diagnosis.

The availability of accurate and rapid diagnostic tests is necessary not only for control programs but also for prognosis, monitoring, and epidemiology of BVDV infection. Another area in which accurate BVDV diagnosis is important is the presence of BVDV or its antibody in biologics of bovine origin, such as fetal bovine serum, cell cultures grown in BVDV-contaminated fetal bovine serum, and even stocks of viruses prepared in BVDV-contaminated cell cultures. For example, Nakamura et al. (1993) isolated noncytopathic (ncp) BVDV from three different stocks of cytopathic (cp) BVDV using a reverse plaque formation method based on intrinsic interference phenomenon.

As stated above, fetal bovine serum (FBS) and calf serum are extensively used in cell cultures as nonspecific nutrients and are often contaminated with BVDV (Bolin et al., 1991; Potts et al., 1989). The use of contaminated sera can result in contami-

nation of cell cultures affecting the production of biological reagents and the results of diagnosis. Bolin et al. (1994) examined 41 cell lines in the ATCC collection and found viral antigen or RNA in 13 of them by immunohistochemistry (IHC) and RT-PCR. The use of contaminated cells may result in contaminated vaccines, which may lead to seroconversion or disease in the vaccinated animals or humans (Levings and Wessman, 1991; Wessman and Levings, 1999). Hypervirulent outbreaks of BVDV in the Netherlands and Italy were attributed to BVDV contamination of bovine herpesvirus type 1 marker vaccine (Falcone et al., 1999). The use of contaminated serum or cell cultures may also interfere with the diagnosis of viral infections by interfering with the growth of other viruses.

Genital tracts are often obtained from abattoirs to harvest cumulus-oocyte complexes and coculture of feeder cells (oviduct epithelial cells and granulosa cells) for use in vitro bovine embryo production systems. A certain number of these tissues are likely to be contaminated with BVDV and their use represents a risk of BVDV transmission. Hence, all materials of animal origin used in the production of bovine embryos for in vitro fertilization should be screened for BVDV (Givens et al., 2002).

It is important, therefore, to continually monitor FBS, cell cultures, seed viruses, and live vaccines prepared in cell cultures for BVDV. Both cp and ncp strains of BVDV should be looked for although most of the contamination is associated with ncp strains, which infect cells without morphological alterations, inducing problems that arise after several cell generations. Gamma irradiation, exposure to ultraviolet light, and inactivation with beta propiolactone have been used to cure BVDV from FBS (Zabal et al., 2000).

A number of different tests are available for the detection of antigen, antibody, and viral components (antigen and nucleic acid) of BVDV. Each method has its advantages, disadvantages, and applicability. Factors that can affect the efficiency of a particular diagnostic method include antigenic and/or genetic diversity of the virus, variation in virus load, and interference from maternal antibodies obtained through colostrum. Methods using monoclonal antibodies (Mabs) can be used to differentiate pestiviruses (BVDV, border disease virus of sheep, and classical swine fever virus of pigs). Monoclonal antibodies prepared against NS2-3 protein are panpestivirus because they recognize highly conserved epitopes (Mignon et al., 1992). Methods are also available to classify BVDV into subtypes 1a, 1b, and 2.

DIRECT ANTIGEN DETECTION

Methods for direct antigen detection in clinical samples are rapid and are often as sensitive as some of the other methods. However, the presence of viral antigen in tissues is often not associated with lesions, particularly in subclinical and persistent infections. When lesions appear, they are seen primarily in lymphoid tissues, where the presence of viral antigen is associated with lymphoid depletion (Liebler-Tenorio et al., 2002). In persistent infections and mucosal disease, virus can be isolated from almost all tissues. Similarly, infection with a virulent BVDV 2 usually results in a widespread dissemination of viral antigen in the host tissues. The tests that can be used for direct antigen detection in fresh, frozen, or fixed tissues include ELISA, IHC (including immunoperoxidase staining of peripheral blood leukocytes and skin biopsies), and immunofluorescence.

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In this procedure, cryostat sections of fresh tissues or smears of buffy coat cells are stained with fluorescein-conjugated anti-BVDV antibody and then examined under a fluorescent microscope. The presence of apple green fluorescence indicates a positive test. This method is known as direct fluorescent antibody (DFA) or direct immunofluorescence test. Although DFA on buffy coat cells has been advocated for the detection of PI animals (Bezek et al., 1988), in our hands, the technique was not very successful (Werdin et al., 1989a).

IMMUNOHISTOCHEMISTRY OF PERIPHERAL BLOOD LEUKOCYTES

An indirect immunoperoxidase test has been used to detect BVDV in smears of buffy coat cells (Saino et al., 1994). Deregts and Prins (1998) developed a Mab-based immunoperoxidase monolayer assay (IPMA) for the detection of BVDV and compared it with a bovine polyclonal antibody (Pab)-based IPMA. A pool of five Mabs (four Mabs against BVDV 1 and one Mab against BVDV 2) was employed. These Mabs were chosen because of their broad cross-reactivity, antigenic avidity, reactivity to different BVDV proteins, and lack of competition for binding sites or binding to unusual BVDV isolates. The Mab-IPMA was found to outperform the Pab-IPMA in staining, ease of reading test results, and relative sensitivity.

IMMUNOHISTOCHEMISTRY OF SKIN BIOPSIES

Immunohistological testing of skin biopsies (ear notch samples) has been used to detect PI animals.

The technique can also be applied to dead animals using thyroid gland, skin, oral mucosa, esophagus, and abomasum as samples for IHC (Thur et al., 1996). The IHC staining of formalin-fixed, paraffin-embedded tissues is an efficient method for the detection of BVDV and is often considered to be better than histopathology (Haines and Ellis, 1994; Hewicker-Trautwein et al., 1995). In a study of 41 cell lines for the presence of BVDV antigen or RNA, Bolin et al. (1994) found an excellent correlation between IHC and RT-PCR. The presence of colostrum-derived antibodies did not interfere with IHC of skin biopsy (Grooms and Keilen, 2002).

To compare IHC with virus isolation (VI), Grooms and Keilen (2002) screened samples from 332 calves. Formalin-fixed skin biopsy samples were stained by IHC and virus was isolated from buffy coat cells. Six calves were positive by both techniques. One was VI-positive and IHC-negative due probably to acute infection since IHC does not detect acute infections but VI does (Ridpath et al., 2002). In another study, skin from 41 of 42 calves, known to be PI by repeated virus isolation, were found to be positive by IHC (Njaa et al., 2000).

The Mab used for IHC should be chosen carefully because only one of 32 Mabs against BVDV proteins and glycoproteins was able to detect BVDV in formalin-fixed tissues by IHC (Haines et al., 1992). This Mab (designated 15C5) is widely employed to detect viral antigen by IHC (Baszler et al., 1995; Ellis et al., 1995).

ENZYME-LINKED IMMUNOSORBENT ASSAY

Many antigen-capture ELISAs have been developed for the direct detection of BVDV antigen in buffy coat cells, serum, and ear notch samples. The basic principle consists of the use of monoclonal antibodies to capture viral antigen followed by detection of antigen-antibody complex with enzyme-conjugated antibody (Bottcher et al., 1993; Ludeman and Katz, 1994). A test that can detect all currently circulating strains of BVDV is the most desirable. The most commonly used antigen capture ELISA (AC-ELISA) uses Mab directed against a conserved antigenic domain of a nonstructural protein (NS2/3) of pestiviruses. The captured antigen is then detected with a pestivirus-specific polyclonal peroxidase conjugate (Gottschalk et al., 1992). Serum is a good sample for the detection of PI animals by antigen-capture ELISA. Acute animals are rarely detected because the virus is present in the blood of an acutely infected animal only for a short time. Monoclonal antibodies against NS2-3

have been used in antigen capture ELISA tests. These tests have been found to yield results comparable to those of virus isolation (Sandvik and Krogsrud, 1995; Greiser-Wilke et al., 1992). Entrican et al. (1995) developed a double Mab ELISA for the detection of viral antigen in blood samples. Two Mabs against p125/p80 were used to capture viral antigen from blood and another two Mabs were used to detect the captured antigen. The Mab ELISA was found to be more sensitive than Pab ELISA.

VIRUS ISOLATION

The most reliable method for the detection of BVDV infection has been the isolation of BVDV in cell cultures followed by identification of the viral isolate by immunofluorescence or immunoperoxidase monolayer assay (IPMA; Meyling, 1984; Werdin et al., 1989a) or RT-PCR (Ridpath et al., 2002). During viremia, virus can be isolated from nasal discharge, PBL, lungs, and feces. Semen, blood, serum, fetus, and feces can be used for virus isolation. However, the presence of anti-BVDV antibody may interfere with virus isolation from serum and buffy coat samples.

Many different cells of bovine origin support the growth of BVDV but bovine turbinate (BT) cells are the most widely used for virus isolation because they are more sensitive to BVDV-induced cytopathic effects, which makes it easier to differentiate cp from ncp strains.. A comparative study was carried out to determine the susceptibility of five different cell types to BVDV. The cell systems used were swine testicle (ST), mink lung (ML), bovine turbinate (BT), porcine kidney (PK15), and equine dermal (ED) cells. The titers obtained on day 8 postinfection were $10^{1.13}$, $10^{3.25}$, $10^{4.13}$, $10^{0.00}$, and $10^{0.00}$, in ST, ML, BT, PK15, and ED cells, respectively, indicating that BT and ML cells are optimal for the propagation of BVDV (Onyekaba et al., 1987). In another study, primary bovine embryo kidney (pBEK) cells and two cell lines originating from bovine embryonic trachea (EBTr) and buffalo lung (IMR-31) were found to be equally susceptible to BVDV (Ferrari, 1985).

Isolated virus can be confirmed by DFA (direct fluorescent antibody assay), immunoperoxidase, antigen capture ELISA, or RT-PCR. For DFA, infected cells are rinsed in PBS and fixed in anhydrous acetone for 10 minutes. Fluorescein (FITC)-conjugated anti-BVDV conjugate is allowed to react at 37°C for 25 minutes. After rinsing lightly, the infected cells and soaked in pH 9 carbonate-bicarbonate buffer

with 0.05% Tween-20 for 10 minutes. The stained cells are examined under a fluorescent microscope. Positive cells are characterized by the appearance of apple green fluorescence. In immunoperoxidase tests, a peroxidase-labeled conjugate is used instead of an FITC-labeled one and the stained cells are examined by light microscopy.

Saliki et al. (1997) compared two techniques for the identification of BVDV isolated in cell cultures. Serum samples were inoculated in indicator cells contained in 96-well microtiter plates followed by immunostaining of infected cells with a pool of Mabs using either immunoperoxidase monolayer assay (IPMA) or the monolayer enzyme-linked immunosorbent assay (M-ELISA). In IPMA, positive samples developed a red intracellular precipitate; a yellow color appears in solution in m-ELISA. The optimal time for staining was determined to be 4 days postinoculation. Although both tests were sensitive and specific, the authors preferred M-ELISA because of its rapidity and greater objectivity. The IPMA virus isolation-immunoperoxidase test (IPX) was also found to be sensitive for the detection of BVDV in another study (Castro et al., 1997). The increased sensitivity of these tests is due to virus isolation component associated with them (Saliki et al., 1997). Pooled Mabs are often used in IPMA and ELISA to detect BVDV after amplification in cell cultures because all Mabs do not give equivalent results. For example, some of the E2-specific monoclonal antibodies generated with BVDV 1 are cross-reactive with BVDV 2 and others are not (Deregt and Prins, 1998; Ridpath et al., 1994).

ANTIBODY DETECTION

An indirect measure of virus infection is the detection of virus-specific antibodies in the sera of animals. Unfortunately, it is often difficult to differentiate among antibodies produced in response to acute infection, vaccination, or transfer of maternal antibodies from dam to offspring. In cattle, calves are usually born without antibody but seroconvert after colostrum consumption. These passive antibodies wane after 3–8 months. Hence, the presence of antibody in colostrum-deprived calves can be due only to active infection (either in utero or postnatal) or vaccination. Seroconversion of sentinel animals can be used as an evidence for possible exposure to PI animals. Many tests are available for the detection of anti-BVDV antibodies—namely, virus-neutralization (VN), indirect immunofluorescence (IIF) assay, indirect immunoperoxidase (IIP), and ELISA tests (Muvavarirwa et al., 1995).

VIRUS NEUTRALIZATION TEST (VN)

The virus neutralization (VN), also known as serum-neutralization (SN), is considered to be the gold standard test for the detection of anti-BVDV antibodies and is used worldwide (Rossi and Kiesel, 1971). In this test, twofold serial dilutions of serum sample are incubated with a constant amount (200–500 TCID₅₀) of the virus for 1 hour followed by the addition of indicator cells. The test is read after 4–5 days of incubation at 37°C. The highest dilution of the serum that inhibits virally induced cytopathic effects in approximately 50% of inoculated cells is considered to be the antibody titer of the serum. The test can be used for the detection of antibodies against BVDV 1 or BVDV 2 depending upon the virus used in the test. In most situations, cp strains of BVDV are used in the test so that the presence of neutralizing antibodies can be detected by inhibition of viral infectivity as detected by the absence of viral cytopathology. However, the test can also be used with ncp strains, in which case the inhibition of viral infectivity is measured by immunoperoxidase staining of infected cells (Fulton et al., 1997).

Cross-neutralization tests can be used to characterize antigenic differences among pestiviruses (Dekker et al., 1995), and titers due to active infection can be differentiated from vaccination titers by demonstrating a fourfold rise in antibody titers using paired serum samples. Virus neutralizing antibodies usually appear 3–4 weeks after infection and persist for years. Titers induced by vaccination may also persist for a long time (Oguzoglu et al., 2003). Passive antibodies decline at 105–230 days (but may persist for more than a year).

ENZYME-LINKED IMMUNOSORBENT ASSAY

Various ELISA tests have been developed for the detection of anti-BVDV antibodies in serum samples. The antigens used in ELISA tests include whole virus antigen, nonstructural protein, monoclonal antibodies, and peptides. Several factors can influence the results of an ELISA test—e.g., antigen, conjugated antibody, test sample, etc. (Schrijver and Kramps, 1998). The procedure used to prepare whole virus antigen can also affect the specificity and sensitivity of the ELISA test. For example, Pilinkiene et al. (1999) found that antigens prepared by mild treatment showed the most specificity and activity. Cho et al. (1991) prepared antigen from MDBK-grown BVDV. The antigen was solubilized

with MEGA-10 (decanonyl-N-methylglucamide) followed by the removal of hydrophobic proteins with Triton X-100 treatment. Compared to VN, this test was 100% specific and 97% sensitive. Moennig et al. (1991) described the development of an ELISA using the nonstructural protein p125/80 of BVDV as antigen. The results were comparable to those obtained by the VN test.

The specificity of serodiagnosis has been enhanced greatly by the use of monoclonal antibody in competitive ELISA systems, and further improvements are possible with the use of defined antigens derived by recombinant DNA techniques (Haines and Ellis, 1994). Lecomte et al. (1990) prepared a panel of monoclonal antibodies that recognized 80 kDa (NS3) antigen of cytopathic BVDV strains. A competitive ELISA was developed using monoclonal antibodies and recombinant protein expressed in *Escherichia coli* as a fusion protein with β -galactosidase. Beaudeau et al. (2001) developed a blocking ELISA using monoclonal antibody against NS2-3 and used this test for mass screening of milk and serum samples with sensitivity and specificity of approximately 97% as compared to VN.

Langedijk et al. (2001) developed a solid phase antibody ELISA using peptides deduced from the C-terminal end (residues 191–227) of pestivirus envelope protein E^{ms}. This ELISA was cross-reactive for several types of pestiviruses and could be used for general detection of pestivirus antibodies. To detect type-specific antibody, a liquid phase ELISA using a labeled specific CSFV peptide, and an unlabeled BVDV peptide (to block cross-reactivity) was used. This test can potentially be used for the differentiation of vaccinated animals from infected ones if vaccination is based on another envelope protein (E2).

A single serum dilution can be used in an ELISA test to quantitate antibodies. Graham et al. (1997) standardized a commercial ELISA test for detection of serum antibodies to BVDV so that a single serum dilution could be tested and the results expressed quantitatively using a standard curve. Various dilutions of known sera were tested and their endpoint titers calculated by an algebraic method directly from a plot of each titration series and also from a regression line fitted to this plot.

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Indirect immunoperoxidase and indirect immunofluorescence tests have also been used for the detection of anti-BVDV antibodies. However, ELISA and VN tests appear to have replaced them.

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR) ASSAYS

Several molecular diagnostic tests have been described for the detection of BVDV infection. Lewis et al. (1991) used ³²P end-labeled, synthetic oligonucleotide probes as a tool for the detection of BVDV RNA. A probe originating nearest the 5' end of the viral RNA detected 86% of the 22 viral isolates tested. Brock (1991) compared DNA dot blot hybridization and RT-PCR for the detection of BVDV in serum and blood samples. RT-PCR was found to provide clearer identification of PI animals than DNA hybridization.

Due to its high sensitivity, RT-PCR is considered as an alternative to current standard methods for detecting BVDV especially in pooled samples such as bulk tank milk. Because the nucleotide sequence of BVDV is highly variable, it is important to carefully select and test primers (Ridpath et al., 1993). The detection of virus by RT-PCR has been found to be more sensitive and rapid than virus isolation. In addition, contrary to virus isolation, RT-PCR is not affected by the presence of antibodies in serum samples. Weinstock et al. (2001) were able to detect the presence of viral RNA when a single viremic sample was pooled with 100 BVDV-negative sera. Horner et al. (1995) compared antigen capture ELISA, RT-PCR, and virus isolation-IPMA. RT-PCR was the most sensitive and ELISA was the least sensitive. The use of RT-PCR is not without disadvantages, however. For instance, the test does not differentiate between nucleic acid from live or inactivated virus and may yield false positive results. In a study by Givens et al. (2002), RT-PCR was positive for BVDV for fetal bovine serum and primary uterine tubal cells used for in vitro embryo fertilization. However, no virus was detectable by virus isolation, and the recipients of washed embryos did not seroconvert.

The prolonged stability of viral nucleic acid as compared to the virus itself has led to a simple method for the collection, storage, transport, and testing of blood samples. In this procedure, 10 μ l of blood or serum is applied to a Whatman No. 1 paper, the sample is air dried and then tested. Using this procedure, Vilcek et al. (2001) were able to detect viral RNA for up to 6 months by RT-PCR while virus isolation was positive for a few days only.

A prerequisite for the performance of RT-PCR is an efficient and simple method for sample preparation. A study was conducted to compare the efficiency of three commercial kits for RNA extraction.

The most sensitive RT-PCRs were obtained when samples were prepared by acidic guanidinium-isothiocyanate-phenol-chloroform extraction with the Trizol (Gibco) reagent. A kit based on the binding of RNA to silica membrane in a spin column had somewhat lower sensitivity, and the kit using salt precipitation of DNA and proteins was unsuitable for the isolation of viral RNA (Scheibner et al., 2000).

Several single- and two-tube RT-PCR assays have been described for the detection of BVDV RNA in serum, buffy coat cells, and fresh and formalin-fixed tissues (including ear notches). Most of these tests are based on the detection of 5' untranslated region (5' UTR) and E2 gene of BVDV. Many different formats of RT-PCR have been described including nested PCR (RT-nPCR) and multiplex PCR. Both virus isolation and ELISA tests are unreliable in the presence of high levels of antibody in the sample but RT-PCR test is not affected (Zimmer et al., 2004).

For retrospective studies, it may be necessary to isolate and amplify viral RNA from formalin-fixed, paraffin-embedded tissues. Gruber et al. (1993) used proteinase K digestion of deparaffinized tissue sections followed by nPCR. This procedure consistently detected an 803 bp fragment of the gene coding for the nonstructural protein p125 of BVDV and was also able to detect BVDV RNA from fresh brain tissue after 10 days of autolysis. Bhudevi and Weinstock (2003) described a TaqMan test for the detection of BVDV in formalin-fixed tissues. The viral nucleic acid was detectable in freshly fixed tissues as well as tissues that were 7 years old. To determine the effect of temperature on RNA degradation, freshly collected tissues from a PI animal were stored at 4°C or at room temperature prior to formalin fixation. Although there was a mild drop in signal strength, tissues stored at 4°C were positive for up to 1 week prior to formalin fixation, but those stored at room temperature were positive until 74 hours only.

NESTED PCR

Gruber et al. (1994) recommended nested RT-PCR over single-step RT-PCR for archival studies because the former was found to be 100-fold more sensitive than the latter. Deregt et al. (2002) described rPCR (conventional RT-PCR after RNA extraction) and dPCR (direct RT-PCR without RNA extraction) and found that both were comparable to virus isolation.

TAQMAN

Open-tube RT-PCRs, in which PCR product is identified by agarose gel electrophoresis, are subject to

contamination with amplicons and are not suitable for high throughput of samples. Several one-tube, real-time formats of PCRs have been developed to minimize sample manipulation. These assays utilize fluorescent signals from oligonucleotide probes to detect amplification of nucleic acid. Hamel et al. (1995) described a simple, one-tube RT-PCR for the rapid detection of BVDV. Total RNA was extracted directly from whole blood and tissue samples. Drew et al. (1999) also described a single step, single-tube RT-PCR test to detect the presence of BVDV in somatic cells from bulk milk samples. The test was highly specific and sensitive, detecting one PI animal in a herd of 162 lactating animals.

Bhudevi and Weinstock (2001) developed a single-tube fluorogenic RT-PCR assay that was found to be 10–100fold more sensitive than the two-tube TaqMan and the standard single-tube RT-PCR assays. They were also able to differentiate between BVDV 1 and BVDV 2 by using two different gene-specific labeled fluorogenic probes for the 5' UTR region. A single-tube RT-PCR, using panpestivirus 324/326 primers targeting the 5' UTR region was developed by Rossmanith et al. (2001).

Mahlum et al. (2002) reported a closed-tube format of nucleic acid amplification and detection. For the development of TaqMan RT-PCR, the forward and reverse primers to 5' UTR (El-Kholy et al., 1998) were lengthened, and degeneracy at specific nucleotide sites was included to enable them to anneal to a wider range of BVDV strains. These primers (forward: 5'-GGGNAGTCGTCARTG-GTTTCG-3'; reverse: 5'-GTGCCATGTACAGCAGAGWTTT-3') amplify a fragment of approximately 190 bases in BVDV types 1 and 2. The TaqMan probe (5'-6-FAM-CCAYGTGGAC-GAGGGCAYGC-TAMRA-3') included a 6-FAM (6-carboxy-fluorescein) fluorescent reporter molecule at the 5' end and a TAMRA (6-carboxy-tetramethyl-rhodamine) quencher molecule at the 3' end. This test was found to be more sensitive than virus isolation and IPMA in detecting BVDV in sera and more sensitive than virus isolation and IHC in detecting BVDV in tissue samples. This test is now routinely used at the Minnesota Veterinary Diagnostic Laboratory and is considered to be rapid, sensitive, and specific.

STRAIN DIFFERENTIATION

The antigenic and genetic variability of BVDV isolates has direct implications on sensitivity of BVDV diagnosis. The diagnostic tests (such as ELISA and RT-PCR) should detect all prevailing strains of

BVDV in the area. A number of investigators have developed RT-PCRs for the identification and differentiation of BVDV and classical swine fever virus. Sullivan and Akkina (1995) developed an nPCR for the identification and differentiation of BVDV 1, BVDV 2, and BDV. Ridpath et al. (1994) designed a PCR to differentiate between types 1 and 2 of BVDV. Later, Ridpath and Bolin (1998) described a differential PCR (based on phylogenetic analysis of 5' UTR) for the segregation of types 1a, 1b, and 2 of BVDV. Harpin et al. (1995) used PCR followed by restriction endonuclease analysis of PCR product to type BVDV. Single-strand conformation polymorphism (SSCP) analysis of polymerase chain reaction (PCR) products, a genetic screening technique for rapid detection of nucleotide substitutions in PCR-amplified genomic DNA or cDNA, has also been employed to identify and differentiate among BVDV isolates (Jones and Weber, 2001).

Fulton et al. (1999) developed a 2-step RT-PCR assay for typing ruminant pestiviruses—e.g., BVDV 1, BVDV 2, and border disease virus (BDV). The first PCR (consensus PCR) detected all three viruses. The amplification of the consensus PCR product by a second (nested) PCR, which uses type-specific primers, resulted in the differentiation of all three viruses. Gilbert et al. (1999) described a nested multiplex PCR for genotyping of BVDV with or without RNA extraction.

Letellier and Kerkhofs (2003) developed a quantitative real-time PCR for the detection and genotyping of BVDV in genotypes 1 and 2. They used a primer pair that was specific for highly conserved regions of the 5' UTR and two TaqMan probes labeled with FAM and VIC. The probe sequences differed by three nucleotides, allowing differentiation between genotypes 1 and 2. Using this procedure they detected 1000 and 100 copies of BVDV 1 and BVDV 2, respectively.

MILK AS A DIAGNOSTIC SAMPLE

Screening of bulk milk samples for antibody or antigen is a promising tool for the detection of PI animals. The milk is centrifuged to remove fat, and undiluted skim milk is tested most commonly in an ELISA test. Because of the ease of obtaining sample, milk sampling is preferable in large scale epidemiological studies and for herd screening. According to the scheme of Alenius, milk samples can be divided into four classes on the basis of antibody levels. Samples belonging to class 0–1 are rarely virus-positive and those in class 2–3 are likely to be positive (Meier et al., 2003). Commercial

ELISAs detecting antibodies against BVDV have been used as a screening test (Bottcher et al., 2003; Meier et al., 2003; Sandvik, 1999). Niskanen et al. (1991) found an excellent correlation between the level of antibodies in the bulk tank milk as detected by an indirect ELISA and the prevalence of BVDV antibody-positive cows. Analysis of bulk milk samples for BVDV antibodies is now routinely used in Scandinavian countries as a tool in the diagnosis of BVDV infections in dairy herds.

To minimize the number of animals tested, Houe (1994) conducted a study to determine whether 10 young stock (8–18 months of age) from a herd can be used as a reliable screening test. The herds were divided into “slightly” to “heavily” infected if less than 3 or more than 8 samples, respectively, were positive for BVDV antibody. No PI animal was found in 24 slightly infected herds and 5 of 18 heavily infected herds were positive for PI animals in a follow-up whole-herd test. In addition, bulk tank milk titers were also generally higher in heavily infected herds.

RT-PCR assay to screen bulk milk samples for BVDV has proven to be a sensitive and economic method for the detection of a single PI animal within a group of several hundred cows (Renshaw et al., 2000) because virus titers are usually higher in milk than in serum samples (Radwan et al., 1995). In this procedure, viral RNA is extracted from somatic cells purified from whole milk using a guanidinium isothiocyanate and phenol/chloroform extraction method. Oligonucleotide primers are selected from the 5' UTR and NS3 region of BVDV genome. RT-PCR of somatic cells was fifteenfold more sensitive than virus isolation, and the presence of antibodies did not interfere with the test. Using RT-PCR on milk samples, Drew et al. (1999) were able to detect one of 162 PI animals. In a comparative study, BVDV could be detected by both virus isolation and RT-PCR when milk from a single PI animal was diluted 1:600 with milk from a herd of BVDV-negative animals. The correlation between the two assays was 95.9% (Renshaw et al., 2000).

SCREENING FOR PERSISTENTLY INFECTED (PI) ANIMALS

The PI animals are considered “virus factories” for their herdmates. They shed large amounts of virus throughout their lifetime and hence should be identified and removed from the herd as soon as possible. After removal of PI animals and cleaning of the herd, all “brought-in” stock should be isolated unless found to be BVDV-free. Although PI animals are an important source of virus on a farm (Duffel

and Harkness, 1985), immunocompetent animals that undergo acute infections can also be a significant source of the virus and should not be ignored. Most of the diagnostic tests are designed and validated based on the detection of PI animals. Since acute infections may also lead to positive reactions from samples of serum, buffy coat, semen, and skin biopsy, it is important to distinguish between acute and persistent infections (Njaa et al., 2000; Ridpath et al., 2002). Confirmation of persistent infection can be done by obtaining positive results from two different serum samples taken 3–4 weeks apart.

Immunohistochemical staining of ear notch samples from calves is considered an effective method to detect PI animals. The ear punch samples are easy to obtain and are relatively stable, and the test is not affected by the presence of maternal antibodies. However, sample pooling is not possible. In addition, tests based on the detection of viral antigen and nucleic acid (IHC and RT-PCR) do not yield a viral isolate that can be further characterized and studied. In a landmark study, Njaa et al. (2000) used IHC staining of formalin-fixed, paraffin-embedded skin biopsy specimens to detect the presence of PI animals. Of the 42 samples that were shown to be persistently infected by repeated virus isolation, 41 were positive by IHC of skin biopsy. Based on the site of immunohistochemical staining, these authors were able to distinguish between persistent and acute infections. Staining in PI animals was most pronounced in keratinocytes, hair follicle epithelium, matrix cells of the hair bulb, and the dermal papilla; in acute infection, small foci of staining were confined to the nonfollicular epidermis and follicular ostia (Njaa et al., 2000).

Buffy coats (PBL) have been used for the isolation of BVDV for PI detection. In conventional method of buffy coat isolation, heparanized blood is sedimented, the plasma layer containing mononuclear and polymorphonuclear leukocytes is aspirated, and then centrifuged at 160 $\times g$ for 5–10 minutes. The cell pellet is suspended in 0.5–1.0 mL of Eagle's MEM followed by inoculation in cell cultures for virus isolation. To maximize leukocyte yield, Ficoll-Paque/Macrodex (F-P/M) is often used. In this procedure, heparanized blood is diluted 1:1 with saline solution and layered on FP solution in a ratio of 3:1 and then centrifuged at 400 $\times g$ for 30 minutes at room temperature. The mononuclear cell fraction lays on top of the FP gradient. This layer is removed, washed twice in MEM followed by resuspension in 2 mL of MEM containing 5% fetal bovine serum (Howell et al., 1979).

Serum is sometimes preferred as a specimen for virus isolation because the virus survives longer in serum and because serum can be collected and stored easily as compared to buffy coat cells (Saliki et al., 2000). However, the presence of antibodies in serum may interfere with virus isolation. In some instances, pooled samples of serum may be the specimen of choice. Serum pools are used in the voluntary control program initiated in the German federal state Saxony-Anhalt in 2002 (Gaede et al., 2003). Initially, pools of sera were tested with real-time RT-PCR in a Light-Cycler system. Individual PI animals in positive pools were detected by using commercial E^{ms}-antigen-ELISA. The prevalence of PI animals was found to be 0.2% during a 3-year period.

A prenatal test that can detect PI animals is not available although fetal fluids obtained percutaneously from late stages of gestation have been tested by virus isolation (Callan et al., 2002). Unfortunately, the procedure is labor-intensive and is not very safe. Lindberg et al. (2001) used an indirect ELISA to identify dams pregnant with PI calves and claimed good sensitivity and specificity after 7 months of gestation.

Some practitioners use paired serology to screen for PI animals. The procedure is to bleed a herd, identify animals with no or low VN titers, revaccinate, and rebleed the whole herd. The animals that do not seroconvert in response to vaccination or that die from mucosal disease are considered to be PI animals. It must be emphasized, however, that this is not a reliable method to screen for PI animals; if the vaccine strain is sufficiently different from the persisting strain, the PI animal may seroconvert to the vaccine strain and be declared non-PI although it still is persistently infected (Werdin et al., 1989a, 1989b; Ferreira et al., 2000). Such animal will not be removed from the herd and will continue to be a source of the virus for its herdmates. Other factors can also result in failure to detect PI animals—e.g., maternal antibodies may interfere with virus isolation, virus load may not be enough for detection, and detection by PCR and ELISA may not be successful if the test is not universal.

SUMMARY AND CONCLUSIONS

A tentative diagnosis of BVDV infection can often be made on the basis of history, clinical signs, and postmortem examination. For definite diagnosis, however, laboratory investigations using rapid and reliable tests are necessary. The methods for the detection of BVDV infections have greatly improved recently; several methods are available for the detec-

tion of virus, antibody, or viral components (antigen and nucleic acid). High throughput methods are now available for whole herd screening to identify and remove PI animals. Bulk milk screening for BVDV or anti-BVDV antibody is used to identify infected herds in Sweden. However, this procedure may not be successful in countries with high cattle densities and in which vaccination is permitted.

Bulk milk and serology of young animals by ELISA test is good. Herds with no or low antibody titers in milk and negative serology in young animals are considered free of PI animals. On the other hand, high titers in milk with or without positive titers in young animals is taken as the evidence that the herd is positive and that further investigation should be undertaken to detect and remove PI animals. The use of serum samples for RT-PCR and of skin biopsies for IHC or RT-PCR has greatly improved BVDV diagnosis. Using these new tools, it should now be possible to consider national control programs for BVDV.

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13

Vaccines

Robert W. Fulton

INTRODUCTION

Bovine viral diarrhea virus (BVDV) infects cattle of different age groups, from fetuses infected during pregnancy through adult cattle. The virus is prevalent in cattle populations worldwide and is responsible for considerable economic losses—e.g., reduction in feed conversion efficiency for meat and milk production, sick cattle, fatal diseases, and costs associated with treatment and prevention including vaccination.

Control programs for BVDV focus on three areas:

- Identification and removal of persistently infected (PI) cattle believed to be the major reservoirs of infection
- Biosecurity measures including prevention of BVDV exposure to herds believed to be BVDV-free through testing of new additions
- Vaccination of postnatal calves to protect them against disease after maternal antibody protection is lost and of heifers/cows to prevent fetal infections causing fetal losses and PI calves.

Vaccination to prevent fetal infections is likely the most important measures to control the occurrence of PI calves.

Since the 1960s, considerable research has been devoted to BVDV vaccines with numerous published studies. Two previous reviews offer extensive coverage, and readers are referred to them for discussion of earlier studies (van Oirschot et al., 1999; Bolin, 1995). In many BVDV vaccine studies, sheep have been used as a model because of their susceptibility to BVDV. The review by van Oirschot et al. (1999) covers numerous BVDV vaccine trials using sheep. This chapter focuses on BVDV vaccines in cattle, especially the efficacy of the vaccines and their impact on BVDV diversity.

Effective vaccines against infectious agents must

account for both antigenic and genetic diversity. There are two BVDV biotypes, cytopathic (cp) and noncytopathic (ncp) based on the presence or absence of visible cytopathic effects in infected cell cultures (Baker, 1995). There are two genotypes of BVDV (BVDV 1 and BVDV 2) based on genomic nucleotide differences, which are detectable by genomic sequence comparisons (Pellerin et al., 1984; Ridpath et al., 1984). Regions of the genome used in detecting genomic differences include the 5' untranslated region (5' UTR), NS (nonstructural protein) region, and E2 (envelope glycoprotein (gp53) region (Collett, 1996). The BVDV 1 genotype can be further separated into subgenotypes BVDV 1a and BVDV 1b (Ridpath and Bolin, 1998). Antigenic differences among these genotypes and subgenotypes are detected by differential virus neutralization tests (Fulton et al., 2003a; Jones et al., 2001).

Antigenic diversity in BVDV is a reflection of sequence diversity in the regions of the genome coding for the envelope protein E2, a glycoprotein (gp 53) E1, and E^{ms} (ribonuclease) protein (Collett, 1996; Potgeiter, 1995). The differences in the 5' UTR are reflected in the E2 region (Pellerin et al., 1994; Van Rijn et al. 1997). Bruschke et al. (1999) reported that the E2 immunogens of BVDV 1a and BVDV 1b may not provide immunity to a heterologous challenge. However, natural infections and the use of killed or modified live virus (MLV) vaccines does induce antibodies to several strains of BVDV including BVDV 1a, BVDV 1b, and BVDV 2 (Fulton et al., 1995; Cortese et al., 1998a; Jones et al., 2001; Fulton et al., 1997; Fulton and Burge, 2000; Fulton et al., 2000a; Fulton et al., 2002a; Grooms and Coe, 2002; Fulton et al., 2003a). However, vaccines containing BVDV 1a were found to induce higher antibody titers to the homologous BVDV 1a strains than to the heterologous BVDV 1b and

BVDV 2 strains. Although BVDV genotype is important in antigenic diversity, this does not appear to be true of biotypes. Studies have shown no differences in antibody titers when comparing cp and ncp strains of BVDV (Fulton et al., 1997; Fulton and Burge, 2000).

The impact of BVDV diversity on clinical forms of the disease is evident when examining BVDV isolates from cases submitted by veterinarians to the diagnostic laboratories (Fulton et al., 2000b). Of the 105 BVDV positive samples (Fulton et al., 2000b), 26 were BVDV 1 cp strains (24.8%), 38 were BVDV 1 ncp strains (36.2%), 10 were BVDV 2 cp strains (9.5%), and 31 were BVDV 2 ncp strains (29.5%). The ncp biotype was isolated more frequently (65.7%) than the cp biotypes (34.3%), and BVDV 1 genotype was more frequently isolated (61%) than BVDV 2 genotype (39.0%). Cattle with respiratory disease had more ncp biotypes than cp and more BVDV 1 genotypes than BVDV 2. In cases of fibrinous pneumonia, more BVDV 1 were isolated than BVDV 2 and of the 41 BVDV 1 isolates, 68.3% were BVDV 1b strains and 31.7% were BVDV 1a (Fulton et al., 2003a). In two studies of over 300 cattle commingled and observed for approximately 5 weeks, BVDV 1b was the most prevalent isolate (Fulton et al., 2002a). The results of these studies indicate that diverse BVDV strains (BVDV 1b, BVDV 1a, and BVDV 2) are found in various BVDV-associated diseases in the cattle population. BVDV 1a, BVDV 1b, and BVDV 2 were isolated from tissues of cattle receiving BVDV 1a vaccines weeks to months prior (Fulton et al., 2000b; Van Campen et al., 2000; and Fulton et al., 2003a). These BVDV strains may have been present due to two possible scenarios: the BVDV vaccinal strains did not induce protective immunity to these diverse strains, these BVDV strains may be PI strains.

VACCINES

Over 160 BVDV vaccines are listed in *the Compendium of Veterinary Products* (2003). These USDA licensed vaccines are based either on BVDV alone or BVDV in combination with bovine herpesvirus-1 (BHV-1), parainfluenza virus type 3 (PI-3), bovine respiratory syncytial virus (BRSV), *Leptospira* spp., *Campylobacter* spp., *Haemophilus somnus*, *Mannheimia haemolytica* and/or *Pasteurella multocida* immunogens. These vaccines meet the requirements for safety, purity, potency, and efficacy by the USDA's Center for Veterinary Biologics (CVB) Code of Federal Regulations Sections 113.311 ("Live virus vaccines," "Bovine virus diarrhea vac-

cine") and 113.215 ("Killed virus vaccines;" "Bovine viral diarrhea vaccine, killed virus"). This information is available at the USDA APHIS CVB website (www.Aphis.usda.gov/vs/cvb/index.htm).

The requirements for efficacy of the killed or MLV vaccines include protection against clinical illness after challenge with virulent BVDV. For MLV vaccines, the challenge occurs at 14–28 days post-vaccination with an observation period of 14 days. For killed vaccines, the challenge occurs 14 days or more after the last dose of the vaccine is given. There is no requirement for the use of a specific BVDV subtype as a challenge in either of these efficacy requirements. In September 2002, the USDA APHIS Center for Biologics Notice No. 02-19 published "Vaccine Claims for Protection of the Fetus Against Bovine Viral Diarrhea Virus." Three categories for label claims were identified: "Aids in the prevention of abortion," "Aids in prevention of persistently infected calves," or "Aids in the prevention of fetal infection" or "Aids in the prevention of fetal infection including persistently infected calves." A product label claim must be supported by research data filed by the vaccine manufacturing company during the licensing process.

BVDV vaccines have been available for use in cattle for over 40 years in the U.S. These vaccines, either MLV or killed, have been an integral part of bovine vaccination programs for beef breeding herd, stocker-feeder cattle for pasture forage or feedlots, dairy cattle, and veal operations. The merits of killed versus MLV vaccines have been debated over time. There are three important issues that have directed attention on the appropriate use of BVDV vaccines. First, in 1993 ncp BVDV strains with enhanced virulence were isolated in Canada from cases of severe acute BVD disease in cattle (Carman et al., 1998). Analysis of the isolates indicated that they were BVDV 2 strains. Cattle not properly vaccinated according to manufacturer's instructions died with severe acute disease and hence efforts were made to increase the usage of BVDV vaccines. Also, the recognition of antigenic differences between BVDV 1 and BVDV 2 indicated the need for protection of cattle against BVDV 2 either by BVDV 1 vaccine or by developing BVDV 2-specific vaccines. The second issue is the recognition that PI cattle, because they shed virus throughout their lifetime, are likely reservoirs of infection for susceptible cattle. This focused attention on the need to control fetal BVDV infections by vaccination prior to exposure. Prior to this, fetal protection studies were not a part of efficacy requirements. The third issue is that the ability

of the vaccines in use from the 1960s through the 1990s to confer protection against BVDV 2 has not been established. The vaccines in use since the 1960s had been, in retrospect, BVDV 1a cp strains, such as the Singer, NADL, and C24V (Oregon) strains. Questions then arose as to whether to accept or prove that BVDV 1 vaccines protected against BVDV 2 when used according to the label or whether BVDV 2 strains should be added to the BVDV 1a vaccines.

VACCINE STRAINS

Virus strains being used in various BVDV vaccines are shown in Table 13.1. In 2002, the genotype BVDV 2 was further divided into subgenotypes, BVDV 2a and BVDV 2b (Flores et al., 2002). Selected BVDV strains have been subtyped (J.F. Ridpath, personal communication, 2003) and those are reflected in the Table 13.1. The vaccines in the U.S. primarily contain BVDV 1a cp strains, although some vaccines do contain BVDV 2 cp strains (refer to Table 13.1). All but four vaccines contain cp biotypes; only two killed and two MLV vaccines contain ncp strains. Of the latter vaccines, one contains a ncp strain, WRL, referred to as BVDV 1 without any reference to the subtype, and another contains a BVDV 1 ncp strain (GL 760) and a subtype is not described. One killed vaccine contains

strain 6309, a BVDV 1 ncp strain whose subtype is not specified. Only one killed vaccine contains ncp BVDV 1b. A dendrogram representing genetic relatedness for reference BVDV strains, including many vaccinal strains, is shown in Figure 13.1. There are several companies in the U.S. that manufacture vaccines or single/multiple immunogens that are marketed by other companies. Thus, vaccines sold under different names may have identical immunogens.

Isolation of BVDV 1b and BVDV 2 from clinic cases cited above demonstrates the need for BVDV vaccines that confer a broad spectrum of complete immunity to these multiple BVDV subtypes. The addition of BVDV 2 immunogen to either MLV or killed BVDV 1a vaccines has been the subject of marketing efforts by certain companies; some other companies, rather than including type 2 immunogens in their vaccine, have submitted data that satisfied licensing requirements allowing a label claim for cross protection against BVDV 2.

MLV VACCINES

The advantages and disadvantages of MLV BVDV vaccines are similar to those of other MLV vaccines. The MLV vaccines require lesser amounts of virus than do killed vaccines because the vaccine replicates in the host to build immunogenic mass. In general, MLV vaccines require only one dose for initial

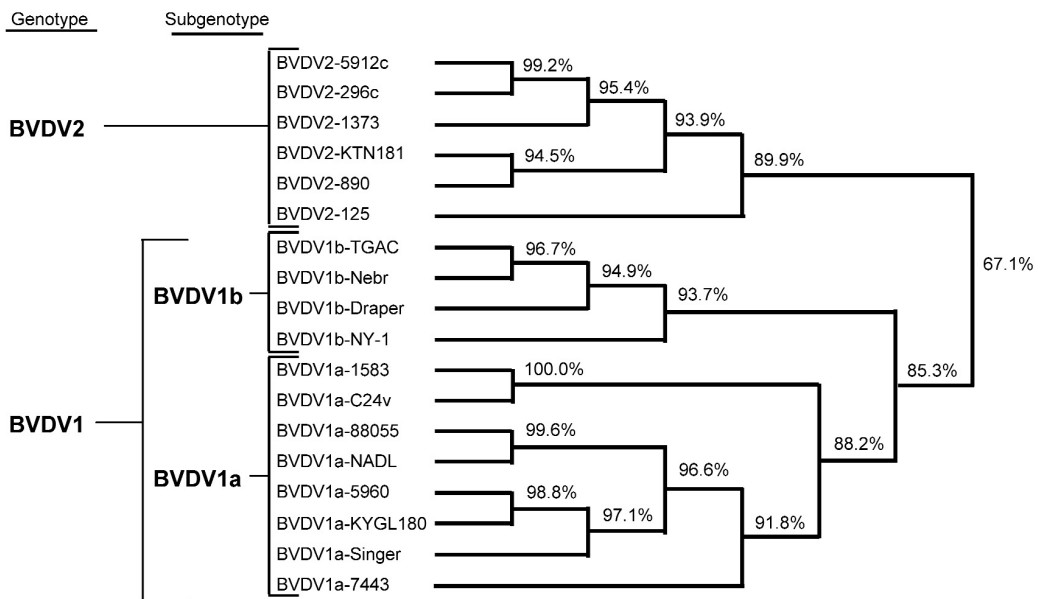


Figure 13.1. Dendrogram representing the relatedness of nucleotide sequences from reference BVDV 1a, BVDV 1b, and BVDV 2 strains.

immunization but do require more rigid handling procedures because the vaccine virus is susceptible to inactivation by chemicals and/or exposure to higher temperatures. Upon administration of MLV vaccine, the BVDV vaccinal strains replicate in the susceptible bovine, resulting in viremia (Cortese et al., 1997; Fulton et al., 2003c; Grooms et al., 1998). The duration of viremia is between 3 and 7 days after which the virus is cleared as the calves develop antibodies (Fulton et al., 2003c). Thus vaccinal strains must be differentiated from field strains if BVDV is isolated from calves within 2 weeks after MLV vaccination. Live virus in MLV vaccine may also cause immunosuppressive effects on leukocyte function of vaccinated cattle (Roth and Kaberle, 1983), potentially rendering them more susceptible to other infections. Another concern is the effect of live vaccine virus on the reproductive tract, because BVDV virus or antigen was detected in the ovaries of heifers receiving an MLV BVDV vaccine up to 30 days prior to testing (Grooms et al., 1998).

A major concern for MLV BVDV use was the observation of a postvaccinal disease following MLV vaccination. Within 1–4 weeks after MLV vaccination, a mucosal disease (MD)–like syndrome occurred in cattle (Bittle, 1968; McKercher et al., 1968; Peter et al., 1967; Rosner, 1968; Lambert, 1968). Postvaccinal MD may result when an animal persistently infected with a ncp virus is exposed to the cp BVDV included in the vaccine. However, vaccination of PI animals with an MLV vaccine will not invariably cause MD. There have been multiple studies whereby PI calves have been given MLV BVDV vaccines with cp strains and no MD resulted (Bolin et al., 1985b; Bolin et al., 1988; Fulton et al., 2003b). Those studies used cp strains that were antigenically different from the PI strains, and the calves developed antibodies to the cp strain in the vaccines. Thus PI animals may not be cleared from herds by vaccination, and failure to develop antibody titers following vaccinations is not a good method to screen for PI animals.

In addition, postvaccinal disease can result from infection with adventitious ncp field strains of BVDV introduced via contaminated fetal bovine serum or cell lines used in vaccine manufacturing. The ncp BVDV is a frequent contaminant of fetal bovine serum (Bolin and Ridpath, 1998; Studer et al., 2002). An example of BVDV contamination of an MLV vaccine occurred when vaccination with a BHV-1 marker vaccine with BVDV 2 contamination caused severe disease beginning 6 days after vaccination with high morbidity on 11 of 12 farms

(Barkema et al., 2001). Signs included nasal discharge, fever, and diarrhea. Necropsy revealed erosions and ulcers of mucosa of the digestive tract.

The ability of BVDV to infect the fetus resulting in abortions, stillbirths, and development defects has caused MLV BVDV vaccines to be contraindicated in pregnant cattle (Orban et al., 1983; Liess et al., 1984). In one study, MLV vaccine containing a BVDV 1a cp strain (C24V) was used in pregnant heifers resulting in fetal infection and disease. In addition there were ncp strains isolated from affected calves diagnosed as PI. The source of the ncp strains was not identified; however, a cp vaccine containing ncp strains is possible. Another study indicated that cp challenge inoculums may give rise to ncp strains (Done et al., 1980). Pregnant heifers were inoculated with a pool of BVDV cp strains (10), and transplacental infections occurred with all ncp strains being isolated, but no cp isolates.

Potentially a vaccine containing a ncp BVDV contaminant could induce a PI calf if the fetus were exposed between days 42–125 of gestation (McClurkin et al., 1984). Fetuses exposed in the last trimester of pregnancy could survive, but may have antibody titers in the precolostral serums after calving. Data suggest that MD was induced after recombination between a ncp BVDV 2 strain with a BVDV 1a cp NADL vaccine strain (Ridpath and Bolin, 1995). The cp strains can cause fetal infections; however, experimental inoculation of pregnant heifers with a cp strain resulted in no PI calves (Brownlie et al., 1989). The potential for a replicating vaccine virus or a contaminating ncp to be transmitted to susceptible contacts must be addressed for any MLV vaccine. Although an MLV BVDV may replicate in a susceptible animal causing viremia, virus may or not be shed. More importantly, there must be sufficient virus shed to infect contacts as indicated by viremia and/or seroconversion. In a recent study (Fulton et al., 2003c) calves vaccinated with each of three BVDV 1a MLV vaccines developed a transient viremia that was cleared after antibodies were induced; however, there was no transmission of the virus to susceptible contact calves in the same pen. The use of ear notch immunohistochemistry (IHC) for BVDV antigen did not detect IHC positives in a preliminary study testing calves after they received MLV BVDV vaccine (Dubois et al., 2000).

KILLED VACCINES

Killed vaccines also have advantages and disadvantages. From a production cost standpoint, killed vac-

cines are expensive because larger amounts of virus are required to prepare each dose of the vaccine as compared to MLV vaccines and there is the added cost of adjuvants. The process of virus inactivation for the production of killed vaccine is likely to also inactivate possible contaminants if any; however, this is not guaranteed unless the final product is tested for replicating virus. Killed BVDV vaccines are generally safer in the pregnant cow, and some vaccination programs advocate killed BVDV vaccines during pregnancy. One disadvantage might be that two doses are generally required for the initial immunization. Both MLV and killed BVDV vaccines have induced antibodies to a wide range of BVDV subtypes, usually resulting in higher antibodies to the specific BVDV subtype(s) in the respective vaccine (Fulton et al., 1997; Fulton and Burge, 2000).

The duration of antibody titers to various BVDV strains was found to vary among different studies. Cortese et al. (1998a) reported that cattle receiving an MLV BVDV 1a (NADL) vaccine induced antibodies to numerous BVDV 1 and BVDV 2 strains detectable through 18 months after vaccination. In other studies, there was a decline in BVDV antibodies by 140 days after initial vaccination (Fulton et al., 1995; Fulton and Burge, 2000). Revaccination at day 140 with either killed or MLV vaccine did induce increased antibodies in calves, especially those with low antibody titers (Fulton et al., 1995 and Fulton and Burge, 2000). This rapid anamnestic response points out that, while antibody titers may decline or disappear, an improved immune response remains in effect. From this standpoint, the true duration of immunity cannot be determined by just measuring serum antibody levels but should rather be determined by challenge with virulent virus.

PROTECTION BASED ON DISEASE FORM

The efficacy of a BVDV vaccine should be measured by three different methods (Van Oirschot et al., 1999). The first is experimental vaccination under controlled conditions followed by direct experimental viral challenge and observation of clinical signs. Secondly, there should be transmission studies to determine whether the vaccine prevents or reduces the transmission of the challenge virus. Finally, field trials of the vaccine are needed to determine protection/cost benefits under production conditions and natural exposure. To truly help dairy and beef cattle clinicians and producers make rational decisions on appropriate vaccine use, the

studies outlined above should go beyond the minimal licensure requirements.

Recently the USDA APHIS CVB began requiring data on file to support label claims. In an ideal setting, methods should be available for veterinarians and the public to analyze the documentation for approved and marketed vaccines. All too frequently, the public's only access to vaccine efficacy information is from marketing materials and advertisements for vaccines. Use of peer-reviewed publications would be an appropriate means to that end for public information. Intellectual property issues should be honored, but the experimental design, results, statistical analysis, and interpretation (discussion) should be available to allow producers and veterinarians to make informed decisions.

MECHANISM OF PROTECTION FOR BVDV

Cattle are capable of mounting both humoral (antibody) and cell-mediated (T-cell) immune responses to BVDV. These responses occur after vaccination or field/natural infection of susceptible, seronegative cattle. As cited above, the vaccines induce antibodies to multiple BVDV subtypes, but antibody titers are generally higher to the vaccine strain and to strains belonging to the same genotype or subgenotype. Numerous vaccine efficacy/immunogenicity studies in which antibody titers have been measured are available in published literature. Unfortunately, in many cases, the authors do not identify the BVDV subtype in either the vaccine, natural infection, or challenge strain. The challenge strain used in the laboratory for a virus neutralization test is also not always known. Our current understanding of antigenic diversity among genotypes and subgenotypes calls for more attention to subtypes in future immunity studies.

The fact that antibodies provide protection is clear from studies that demonstrate passive protection against BVDV challenge in calves fed colostrum containing BVDV antibodies (Howard et al., 1989; Bolin and Ridpath, 1995). Resistance either to disease severity or viral infection/shedding was dependent upon BVDV antibody titers in the sera. Cortese et al. (1998) showed that calves fed colostrum with BVDV antibodies were protected against experimental BVDV. However, high concentrations of maternally derived BVDV antibodies have been shown to block a protective response to MLV BVDV 1a NADL vaccine (Ellis et al., 2001). Kirkpatrick et al. (2001) reported that dairy calves fed colostrum with BVDV antibodies had low BVDV antibody titers of

1:4–1:16 and did not seroconvert to either BVDV 1a or BVDV 2 after vaccination with an MLV BVDV 1a and BVDV 2 vaccine.

Postnatal calves develop T-cell immune responses after vaccination or infection. It is assumed that these calves can develop concurrent humoral antibody and T-cell responses. Trying to determine which arm of the acquired immunity (humoral or cell-mediated) is more important in protection is difficult because in the intact, healthy susceptible animal, it is hard to immobilize one arm of the immune system to determine its role in protection. Beer et al. (1997) demonstrated that immunized and experimentally exposed calves responded with positive cytotoxic T-cell responses, along with high neutralizing antibody titers. More recently, Endsley et al. (2002) reported that calves developed antigen-specific T-cell responses when given either a BVDV 1 MLV or BVDV 1 and BVDV 2 MLV vaccine. Information on humoral antibody responses to BVDV 1 and BVDV 2 was not provided. Using a variety of tests for T-cell immunity and antibodies, the protective immune mechanism was not apparent in calves receiving an MLV BVDV 1 vaccine and subsequently challenged with heterologous BVDV 2 (Cortese et al., 1998b).

Passively acquired BVDV antibodies prevented clinical disease in colostrum-fed calves exposed to virulent BVDV (Ridpath et al., 2003). The serum antibody titers decayed at the same rate as in unchallenged colostrum-fed calves. These same colostrums-fed calves previously challenged were still protected from clinical disease after the serum antibodies had decayed to undetectable levels. A protective response was thus mounted in calves with passive immunity but which was not reflected by antibody titers. According to the authors, cell-mediated immunity may have a role in preventing postnatal disease.

EFFICACY STUDIES

In a review of published studies for both postnatal calves and fetal infection/disease, van Oirschot et al. (1999) cited numerous trials in both sheep and cattle for the prevention of fetal infections and trials in cattle for preventing postnatal infections. In that review, numerous reports were cited indicating the use of sheep as a model for BVDV vaccine development and evaluation. Although sheep are an active, inexpensive BVDV model, this chapter focuses on studies of current North American vaccines in cattle. Selected other studies will be included that address vaccine technology and experimental design, which

have worldwide relevance. Until recently, the impact of BVDV antigenic diversity on vaccines and challenge models had not been addressed. In addition, only recently efforts have been made to study vaccine protection against fetal infections. One of the few early studies was done by Duffel et al. (1984) who reported that immunity to BVDV was induced in heifers prior to breeding, and that their fetuses were protected after challenge at day 100 of gestation (Duffel et al., 1984).

This chapter updates previous published studies with the current designation of subtypes. However, it should be realized that a large database of all known BVDV strains with respect to specific subtype designations does not exist. Experimental studies are available that have evaluated both killed and MLV vaccines. The biotype/genotype in the vaccines until recently was confined to the BVDV 1a cp strains. Likewise, challenge strains have varied, as have experimental challenge methods and parameters for evaluation of clinical illness. In general, the experimental challenge has been relatively soon after the last vaccine dose, usually 14–28 days, and the duration of immunity for the postnatal protection and fetal protection studies has not always and not uniformly been performed. The general consensus for postnatal protection is that there is incomplete protection against clinical signs/disease. Brock and Chase (2000) described an experimental protocol to evaluate fetal protection in cattle by BVDV vaccines.

PROTECTION AGAINST POSTNATAL INFECTION/DISEASE

A combination killed BVDV vaccine containing a BVDV 1a cp strain and a BVDV 1 ncp strain (subtype not designated in Table 13.1) given in two doses induced BVDV antibodies, and vaccinates were protected against clinical illness when compared to controls. The challenge virus, BVDV 1b ncp New York (NY-1) strain, was given 14 days after the second dose. The antibody titers of vaccinates at the time of challenge ranged between 1:16 and 1:128 (mean = 1:42.7). The strain used in neutralization assay was not identified. The protection was not complete because some vaccinates had illness (Talens et al., 1989).

Calves given a MLV vaccine containing the BVDV 1a NADL strain were challenged with BVDV 1b ncp NY-1 strain on postvaccination day 27. None of the vaccinated calves had clinical signs in the 14-day postchallenge period nor did they have rectal temperatures above 39.7°C (Phillips et al., 1975). With the evident need for protection against BVDV

Table 13.1. Strains and types of bovine viral diarrhea virus (BVDV) in modified-live virus (MLV) and killed vaccines.

Vaccine Type and Trade Name	Name of Strain	Genotype/Biotype	Company
MLV			
Arsenal 4.1	GL 760	1 ncp ¹	Novartis
Express 5	Singer	1a cp	Boehringer Ingelheim Vet Medica
	296	2a cp ²	
BoviShield 4	NADL	1a cp	Pfizer Animal Health
Pyramid 4	Singer	1a cp	Fort Dodge Animal Health
Reliant 4	NADL	1a cp	Merial
Frontier 4 Plus	C24V	1a cp	Intervet
	296	2a cp ²	
Titanium 5	C24V	1a cp	Agrilabs
	296	2a cp ²	
Jencine 4	WRL	1 ncp	Schering-Plough Animal Health
Herd Vac 3	Singer	1a cp	Biocor Animal Health
Bovine Viral Diarrhea Vaccine			
	C24V	1a cp	Colorado Serum Co.
Killed			
Elite 4	Singer	1a cp	Boehringer Ingelheim Vet Medica
Horizon 4 Plus	C24 V	1a cp	Intervet
	125C	2a cp	
Master Guard 5	C24 V	1a cp	Agrilabs
	125C	2a cp	
Respishield 4	Singer	1a cp	Merial
Triangle 4 + type II	Singer	1a cp	Fort Dodge Animal Health
	5912	2a cp	
CattleMaster 4	5960	1a cp	Pfizer Animal Health
	6309	1 ncp	
ViraShield 5	KY22	1a cp	Grand Laboratories
	TN 131	2 ncp	
Surround 4	Singer	1a cp	Biocor Animal Health
	NY	1b ncp	

cp = cytopathic; ncp = noncytopathic

¹Information provided by the company.

²Based on sequencing information by Dr. J. F. Ridpath, USDA, ARS, NADC.

2 strains, there have been published studies where BVDV 1-vaccinated cattle have been protected when challenged by BVDV 2. Cortese et al. (1998b) reported a study in which a BVDV 1a NADL vaccinal strain was given to seronegative 10–14-day-old dairy calves and the calves then challenged intranasally with a virulent BVDV 2 24515 ncp strain 21 days later. The antibody titers at the time of challenge

ranged from negative to 1:66 (mean = 1:15) to BVDV 1a NADL and negative to 1:6 (mean = 1:1) to BVDV 2 125C. Vaccinated calves were protected against clinical signs and viremia in the 14-day postchallenge observation period.

A related study by Ellis et al. (2001) indicated that the MLV BVDV 1a NADL vaccinal strain was given to 10–14-day-old or 4-month-old seronegative

calves that were later challenged intranasally with the virulent BVDV 2 24515 ncp strain at 4.5 months of age (3 weeks after vaccination of the 4-month-old calves and 4 months after vaccination of the 10–14-day-old calves). At time of challenge, the neutralizing antibody titers for the 10–14-day-old seronegative calves vaccinated calves were the following: mean of 162 to BVDV 1a NADL (range of 108 to >162) and mean of 108 to BVDV 2 125C (range of 18 to >162). The neutralizing antibody titers at challenge for the 4-month-old calves vaccinated calves were the following: mean of 36 (range of 18–54) to BVDV 1a NADL and mean of 18 (range of 12–108) to BVDV 2 125C. As in the prior study, the calves were protected against clinical illness, and all but one vaccinated calf were protected against viremia.

An MLV vaccine containing the BVDV 1 ncp strain (subtype not identified, Table 13.1) was given to seronegative calves, and the cattle were held for approximately 7 months and then challenged intranasally with the BVDV 2 ncp 890 strain (Dean and Ley, 1999). The neutralizing antibody titers to BVDV 2 890 strain at time of challenge were the following: intramuscular vaccinates, mean of 78 (range 16–362) and subcutaneous vaccinates, mean of 73 (range of 23–181). Clinical signs were reduced in the vaccinated animals as compared to controls in the 21-day postchallenge period. Vaccination eliminated nasal shedding in 87% of the cattle and completely prevented viremia and leukopenia, whereas all unvaccinated cattle shed virus nasally and became viremic. This study was unique in that it demonstrated there was a long duration of immunity (7 months) and the calves were protected against BVDV viremia.

In a study by Howard et al. (1994), two inactivated vaccines were prepared using 11249 NC and Ky 1203 NC using β -propiolactone inactivation. Three doses of each vaccine were given on day 0, week 3, and week 6. The subtypes for these two strains were not identified in the study. Subsequently, studies refer to 11249 NC and Ky 1203 NC BVDV 1a ncp strains (Nobiron et al., 2003; J. Patel, personal communication). Three weeks later the 11249 NC vaccinates were challenged with homologous virus intranasally. The neutralizing antibody titers in vaccinates to 1249 NC was a mean of 1318 ($3.12 \log_{10}$). Nonvaccinated controls became viremic 4–6 days postchallenge and shed virus in nasopharyngeal samples 4–8 days postchallenge; however, the vaccinated animals did not have positive nasopharyngeal swabs or viremia. In the second group, the Ky 1203 NC strain was given on days 0,

weeks 3 and 6, and cattle challenged on week 8 with the heterologous 11249 NC strain. Again, vaccinates were protected against viremia and nasopharyngeal shedding. These studies support the point that killed vaccines, although requiring multiple doses, can protect against viremia and nasopharyngeal shedding.

In another report, Makoschey et al. (2001) found protection by an inactivated BVDV 1 vaccine (Bovilis BVD) against clinical signs including thrombocytopenia after challenge with a BVDV 2 strain. This vaccine contained BVDV 1a cp strain C86 (Patel et al., 2002; J. Patel, personal communication). Calves received 2 doses of the vaccine 4 weeks apart and were challenged 4 weeks later with the BVDV 2 Giessen-1 challenge strain(s), which contained both BVDV cp and ncp biotypes. The antibody titers in vaccinates at time of challenge using the BVDV 2 challenge virus in the serologic test ranged from 5–10 \log_2 (1:32–1:1024). Calves were given the challenge virus both intranasally and intravenously, and the observation period was 14 days for clinical signs. After challenge, unvaccinated cattle developed signs of respiratory disease, diarrhea with erosions and hemorrhage of the digestive tract, and depletion of lymphocytes in lymphatic organs. These signs were absent or markedly less severe in vaccinates. The unvaccinated and vaccinated animals were evaluated for thrombocytopenia and leukopenia for 22 days postchallenge. Beneficial effects of vaccination indicated protection against leukopenia and thrombocytopenia compared to unvaccinated cattle. Vaccinated calves did not shed virus after challenge and had reduced numbers of BVDV isolates from plasma and blood cells. Thus the inactivated BVDV 1 vaccine gave protection against heterologous BVDV 2 infection and disease and reduced viral shedding and viremia.

In another study, two doses of an inactivated vaccine containing BVDV 1 PT 810 strain and BVDV 2 890 strain were given to cattle 28 days apart (Beer et al., 2000). The BVDV PT 810 strain is a BVDV 1c strain (Giangaspero and Harasawa, 1999). Vaccinated and nonvaccinated animals were challenged intranasally with the BVDV 1 PT810 strain 38 days after vaccination. The neutralizing antibody titers in vaccinates at time of challenge ranged from 1:10–1:8192 using the homologous virus BVDV 1c PT 810 in the serotest. Vaccinated cattle had reduced viral shedding in nasal samples, reduced leukopenia, and reduced viremia as compared to controls. This is another example where an inactivated BVDV vaccine given in multiple doses provided protection

after homologous challenge as measured by leukopenia, viremia, and viral shedding.

A Japanese study recently demonstrated protection by a Japanese MLV BVDV vaccine containing BVDV 1 ncp strain 12-43 in calves against a U.S. strain of BVDV 2 (Shimazaki et al., 2003). Four weeks after vaccination, the vaccinated ($n = 3$) and unvaccinated calves ($n = 2$) were challenged intravenously with the BVDV 2 890 ncp strain. The vaccinated calves were seronegative to the BVDV 2 890 strain at the time of challenge; however, the neutralizing titers to BVDV 1 ncp were $>1:64$. The vaccinated calves did not develop clinical signs or fever nor have hematological changes (decreased WBC) after challenge. The vaccinated animals were protected against blood leukocyte infection. The authors suggested that other studies be performed to confirm effectiveness of the vaccine against BVDV 2 strains isolated in Japan.

PROTECTION AGAINST FETAL INFECTION/DISEASE

In the 1990s attention was directed towards developing new vaccines or evaluating existing vaccines to protect the fetus against BVDV infection/disease. Until then vaccine licensure relied on efficacy of protection against acute disease or infection for postnatal exposure of calves. Also label claims by manufacturers required extensive, long-term studies to generate supporting data for the label claim. An inactivated vaccine, Bovidec (C-Vet) containing a Compton prototype virus, was given to heifers as either two or three doses each, 3 weeks apart near the time of breeding (Brownlie, et al., 1995). The vaccine strain in Bovidec vaccine is a BVDV 1a ncp strain (J. Patel, personal communication). Pregnant heifers were exposed intranasally between 25 and 80 days gestation with BVDV Pe 515 NC C1. Vaccinated heifers were protected against viremia postchallenge. There was no evidence of infection in the live calves born to vaccinated dams or in the 2 aborted fetuses from the vaccinates. Thus vaccination gave 100% protection to the 15 fetuses of 15 vaccinated dams. There was, however, fetal infection in 14/15 fetuses in the unvaccinated group as indicated by PI status of newborn calves, infected aborted fetuses, or active immune response prior to birth.

The MLV BVDV 1a NADL vaccine strain was evaluated for protection of fetuses against BVDV 1 strain (BJ ncp strain) (Cortese et al., 1998c). Twelve heifers were vaccinated once, and there were 6 non-vaccinates. Thirty days after vaccination the heifers

were synchronized with a prostaglandin product and then housed with 10 BVDV-free bulls. They remained with bulls for 7 days. Approximately 110 days after vaccination (approximately 75 days gestation) all 18 heifers were challenged intranasally with the BJ ncp strain, a BVDV 1a strain (K.V. Brock, personal communication). Mean neutralizing antibody titers to the BVDV 1a BJ strain and BVDV 1a NADL strain at time of challenge was 1:40 (BJ) and 1:20 (NADL) for 10 vaccinates and 1:10 (BJ) and $<1:5$ (NADL) for 2 vaccinates. The vaccinated animals did not become viremic postchallenge, but 4 of 6 nonvaccinated animals did 6–8 days after challenge. All 6 unvaccinated heifers carried their fetuses to term and gave birth to PI calves as determined by multiple virus isolations. Of the 12 vaccinated heifers, there were 2 with PI calves. Thus fetal protection was demonstrated against homologous BVDV 1 challenge in 10 of 12 (83.3%) of the vaccinated heifers.

The ability of the BVDV 1a MLV NADL vaccine to provide protection against heterologous BVDV 2 was evaluated (Brock and Cortese, 2001) among 19 vaccinated and 6 unvaccinated heifers. Forty-five days after vaccination, the cattle were synchronized with prostaglandin and exposed to 10 BVDV-free bulls for 1 week. Thirty days later, the heifers were examined by ultrasound. All open animals were given a second dose of prostaglandin and were exposed to bulls again for 7 days. Thus challenge appears to have been approximately 4–5 months after vaccination. Mean neutralizing antibody titers in vaccinated animals at the time of challenge was 1:36 to both BVDV 1a (NADL) and BVDV 2 (PA 131). At 75 days gestation, the pregnant heifers were exposed intranasally to PA 131 strain, a ncp BVDV 2 strain. After challenge, 5 of 6 nonvaccinates were viremic (between days 5 and 8), but none of the 19 vaccinates became viremic. The experiment was terminated at 150–180 days gestation, and spleen, thymus, and small intestine of fetuses were collected for virus isolation. All 6 fetuses from the nonvaccinates were PI while 6 of 19 vaccinated dams had PI fetuses. Thus 11 of 19 (57.9%) pregnant heifers given a BVDV 1a NADL vaccine strain were protected against PI. While protection was demonstrated against BVDV 2, it was not complete and was less than protective against homologous BVDV 1 (83.3%) demonstrated previously (Cortese et al., 1998c).

A two-step vaccination protocol, first dose with an inactivated BVDV vaccine (Mucobovin) followed by an MLV BVDV vaccine (Vacaviron), was

evaluated for fetal protection (Frey et al., 2002). The inactivated BVDV vaccine contained BVDV 1b ncp NY-1 strain and a Border Disease virus strain, Aveyronite. The MLV vaccine contained the BVDV 1a cp C24V strain. Heifers were vaccinated with the two-step vaccination program starting with the inactivated BVDV vaccine followed by MLV vaccine 4 weeks later. Neutralizing antibody titers in vaccinates 16 weeks after first vaccination indicated BVDV 1 titers of 1:100–1:600, and BVDV 2 titers of 1:10–1:240. Five months after the first vaccination (at 30–120 days of gestation), the heifers were challenged with a mixture of BVDV 1 ncp 22146/Han 81 and BVDV 2 ncp (strain not identified). A transient viremia was seen 5–9 days after challenge in the nonvaccinated heifers, and BVDV 1 was isolated from 1 heifer and BVDV 2 from the remaining 5. One vaccinate was positive for BVDV 2 on one collection day, but the other vaccinates remained nonviremic. All vaccinated heifers ($n = 9$) gave birth to calves that were not viremic, did not have evidence of congenital fetal damage, and all precolostral sera were BVDV seronegative. The 6 calves born to nonvaccinates had evidence of fetal infection; 1 was stillborn, 1 died 2 days after calving, and 4 were underdeveloped and had signs of ocular defects. Precolostrum serum samples were positive for BVDV 2 by virus isolation. This study demonstrated that a combination of killed BVDV 1b and Border Disease virus vaccine followed by an MLV BVDV 1a vaccine conferred protection against challenge with both BVDV 1 and BVDV 2, and also against transplacental infection with BVDV 2.

A unique study was performed to evaluate the ability of an inactivated BVDV 1a vaccine to protect heifers exposed to PI animals 6 months after initial vaccination (Patel et al., 2002). The use of PI animals to introduce challenge virus mimics the most likely route of exposure under field conditions. Two doses of an inactivated vaccine (Bovilis BVDV) containing BVDV 1a cp strain C 86 were given to 20 heifers 4 weeks apart. Approximately 100 days later, the heifers were synchronized followed by two services. There were 11 vaccinated and 7 unvaccinated animals. Six months after the second vaccination (at about 87 days of gestation) the heifers were challenged. The neutralizing antibody titers in vaccinates at the time of challenge to BVDV 1a ranged from 1:64–1:256. Three PI heifers were introduced into the pen for 2 weeks to initiate challenge. The PI cattle were infected with a BVDV 1a ncp strain (J. Patel, personal communication). All 7 nonvaccinated heifers became viremic, as detected by virus

isolation from serum and/or leukocytes. Only 5 of 11 vaccinated calves were found to be viremic as determined by viral isolation from leukocytes; all were negative for virus in serum. Virus was detected in nasal samples of 5 of 7 nonvaccinates and 2/11 vaccinates. The vaccinated heifers had two abortions (not related to BVDV) and 9 normal calves. These 9 calves were negative for BVDV and BVDV antibodies in precolostral serum. All 7 nonvaccinated heifers delivered BVDV-infected calves. This study demonstrated that two doses of an inactivated BVDV 1a vaccine gave complete protection against homologous viral challenge of pregnant heifers 6 months after vaccination.

The results of these trials evaluating efficacy of BVDV vaccines under challenge conditions indicate protection for both postnatal acute infections/disease and fetal infections. Challenge in many trials was relatively soon after vaccination, 2–4 weeks after last dose of initial immunization, which is presumably at a peak of immunity. In other trials, the cattle were challenged approximately 6 months after vaccination. Interestingly, all of the above studies indicated protection against postnatal acute disease. In some, but not all studies, vaccination eliminated viremia and nasal shedding in postchallenge vaccinated animals. Thus, it may be difficult to totally eliminate nasal shedding from the respiratory tract or viremia (systemic infection) in even highly immune cattle after challenge. It is important that any BVDV vaccine protects against viremia, because viremia indicates the potential for fetal infections. Likewise, elimination of nasal shedding in exposed vaccinated cattle is important to prevent further transmission of BVDV. There appears to be increased interest in protection against different BVDV subtypes, particularly against BVDV 2.

The protection against fetal infection/disease varied from approximately 60–100%. Fetal protection is extremely important to prevent or minimize PI calves. Elimination of PI cattle will likely reduce BVDV transmission. In the two reports using the MLV BVDV 1a vaccinal strain there was protection (83%) against homologous BVDV 1a challenge; however, there was reduced protection against heterologous BVDV 2 challenge, (58%). Although protection was demonstrated, the MLV BVDV 1a vaccine did not provide 100% protection. Thus even though the vaccines may have efficacy demonstrated for licensure, vigilance for PI cattle in vaccinated cattle is important because there may be potential for PI calves born to vaccinated cows/heifers.

Up to this point, this review has focused on killed

and modified live whole virus vaccines produced by classic methods. However, several experimental vaccines for delivering BVDV immunogens have been reported in the literature. Predominantly these vaccines focus on the delivery of the E2 glycoprotein by various methods, including recombinant protein from baculovirus constructs (Bolin and Ridpath, 1996; Bruschke et al., 1997), expression as constituent of recombinant adenovirus (Elahi et al., 1999) and as a DNA construct (Harpin et al., 1999; Norbiron et al. 2003).

GOALS FOR VACCINE DEVELOPMENT

There are significant goals for BVDV vaccines that, if accomplished, would greatly provide better control measures for BVDV. The first involves fetal protection. In order to completely eliminate the birth of PI calves, vaccines need to provide 100% lifetime protection of pregnant heifers/cows against viremia. The second goal involves the prevention of acute infection in nonpregnant animals. Acute BVDV disease remains a problem for both calf and dairy production. Stocker/feeder beef production operations are faced with acute disease caused by BVDV (Fulton et al., 2000a; Fulton et al., 2002a). The goal of vaccination in these animals would be to eliminate clinical signs and virus shedding. In addition, current management practices require a rapid onset of protective immunity for vaccination to be effective. The marketing of beef cattle in North America typically involves assembly of calves from diverse sources, resulting in comingling of calves with unknown vaccination status. Likewise, many cattle of unknown vaccination status are delivered directly to feedlots. Cattle are usually vaccinated against BVDV along with other viral immunogens shortly after purchase at auctions and/or delivery to the feedlot. The period immediately after comingling is critical for exposure to pathogens.

Current BVDV vaccine onset of immunity has not been thoroughly investigated, although efficacy trials have been successful when challenge occurred 14 days after the last dose of initial immunization. The duration of immunity should be established for each vaccine, particularly those with a fetal protection label claim. For example, beef breeding cattle, particularly under range conditions may not always be able to be gathered for vaccinations due to large pastures; hence the need for vaccines inducing durable immunity.

There is an ongoing debate regarding the importance of BVDV vaccines in controlling acute respi-

ratory disease in postnatal calves. A review of field studies on vaccine efficacy in bovine respiratory disease did not demonstrate benefits by BVDV vaccines (Perino and Hunsaker, 1997). In contrast, a later study demonstrated that BVDV immunity was a predictor of illness and performance parameters in feedlot calves (Fulton et al., 2002b). In that study, there was a correlation between higher levels of BVDV 1a antibodies and lower morbidity rate. Calves with low antibody levels to BVDV 1a and BVDV 2 had decreased net value to owners (carcass value minus total feedlot costs). Calves treated twice or more had lower levels of antibody to BVDV 1a than those treated once or not at all. Thus, BVDV immunity appears to demonstrate benefit against disease and for increased profitability. Herds with high morbidity and treatment costs are often shown to follow incomplete vaccination programs.

Finally, there are safety issues that should be addressed. These include assurances that MLV vaccines do not cross the placenta and infect the fetus. The bovine fetus is very susceptible to the field or vaccinal strains of BVDV. In addition, there also needs to be better efficacy studies to heterologous BVDV subtypes.

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14

Management Systems and Control Programs

Robert L. Larson

INTRODUCTION

The management and control of bovine viral diarrhea virus (BVDV) infection in cattle herds must take into consideration two methods of virus transmission: postnatal horizontal transmission and gestational vertical transmission from a viremic dam to her fetus (Meyling et al., 1990). Postnatal horizontal transmission results in a transient infection that is usually mild or subclinical, but can result in severe disease if susceptible cattle are exposed to a virulent strain of the virus (Kelling et al., 2002; Hamers et al., 2000a). In addition, postnatal horizontal transmission can lead to vertical transmission of BVDV, because postnatal horizontal infection is the primary method by which a pregnant dam becomes viremic and subsequently infects her fetus.

PRINCIPAL RESERVOIRS OF BVDV

The primary source of BVDV in postnatal transmission is cattle persistently infected (PI) with BVDV. Persistently infected animals are much more efficient transmitters of BVDV than transiently infected animals because they shed large amounts of virus for a long period of time. Transiently infected animals experience a short period of viremia and shed virus in their body secretions and excretions from day 4–15 postinfection (Brownlie et al., 1987; Duffell and Harkness, 1985). In contrast, PI animals usually have a very high and persistent viremia, and BVDV is shed throughout life from virtually all secretions and excretions, including nasal discharge, saliva, semen, urine, tears, milk, and to a lesser extent, feces (Rae et al., 1987; Brock et al., 1991; Bezek et al., 1995; Brock et al., 1998). Horizontal transmission of BVDV to seronegative cattle has been shown to occur after only 1 hour of direct contact with a PI animal

(Traven et al., 1991). Over-the-fence contact with a PI animal from a neighboring herd can also introduce BVDV into a susceptible herd (Roeder and Harkness, 1986; Miller et al., 2002).

Although transiently infected cattle are far less efficient at transmitting the virus to susceptible in-contact animals (Meyling and Jensen, 1988; Niskanen et al., 2000; Niskanen et al., 2002), the occurrence of seroconversion among assembled cattle without the presence of PI animals indicates that transmission from transiently infected animals does occur (Meyling et al., 1990). In a field study by Moerman et al. (1993) it was found that BVDV infections circulated within a herd over a period of 30 months in the absence of PI cattle and BVDV vaccination, but in the presence of transiently viremic cattle. Horizontal transmission of the virus from either persistently or transiently infected animals to susceptible cattle may be via inhalation or ingestion of virus-containing body fluids (Duffell and Harkness, 1985). Although aerosol transmission over short distances seems likely; the spread of infection is slow or absent when cattle are housed at long distances from PI animals (Wentink et al., 1991).

REPRODUCTIVE EFFECTS OF BVDV

Even mild or subclinical infections of pregnant and susceptible seronegative breeding females may result in conception failure, abortion, or vertical fetal infection. The immune status of the dam, the stage of gestation, and the viral biotype are important factors in determining the result of vertical infection. Transplacental infection occurs with high efficiency in pregnant, seronegative dams (Done, et al., 1980; McClurkin et al., 1984). However, naturally acquired immunity can prevent later fetal infection (Orban et

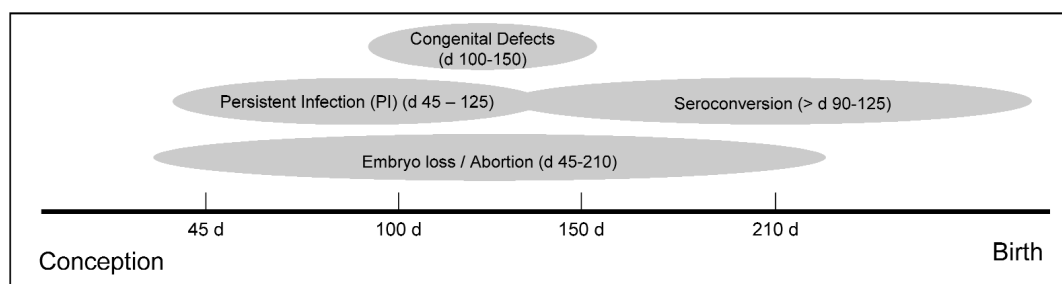


Figure 14.1. The effect of stage of gestation at the time of BVDV infection of susceptible pregnant cows on clinical outcome.

al., 1983). Infection of fetuses can lead to early embryonic death, abortion, congenital defects, stunting, and the birth of either PI or normal calves (Figure 14.1) (Baker, 1987). Fetal infection of susceptible dams with a cytopathic biotype of BVDV early in gestation will usually result in abortion. However, fetal infection with a noncytopathic biotype of BVDV will result either in abortion or, in a certain proportion of animals, the birth of a calf that is immunotolerant and persistently infected with that particular noncytopathic strain of BVDV. Thus, PI cattle are the result of in utero exposure to the noncytopathic biotype of BVDV prior to the development of a competent fetal immune system (Casaro et al., 1971; McClurkin et al., 1984). Age at immune competence in the face of BVDV exposure is variable and has been reported to range from 90–125 days (Casaro et al., 1971; McClurkin et al., 1984; Roeder et al., 1986). If PI fetuses survive to term, they are continually viremic, but immunotolerant to the homologous BVDV (Duffell and Harkness, 1985; Roeder et al., 1986).

In addition to BVDV causing conception failure and abortion, reproductive efficiency can be decreased due to fatal congenital defects following fetal infection between 100 and 150 days of gestation (Duffell and Harkness, 1985). The teratogenic lesions associated with fetal infection with BVDV include microencephaly, cerebellar hypoplasia, hydranencephaly, hydrocephalus, hypomyelination of the spinal cord, cataracts, retinal degeneration, optic neuritis, microphthalmia, thymic aplasia, hypotrichosis, alopecia, brachygnathism, growth retardation, and pulmonary hypoplasia (Baker, 1987).

EXPOSURE OF HERDS TO PI CATTLE

Suckling calves are commonly in contact with the breeding herd and thus come in contact with dams in early stages of pregnancy. As a result, PI suckling calves may be a source of BVDV infection in breed-

ing herds, causing decreased pregnancy percentage, pregnancy loss, preweaning mortality and the induction of PI calves in the next generation (Wittum et al., 2001; Duffell and Harkness, 1985; McClurkin et al., 1984).

Although mortality of PI calves prior to weaning has been reported to be very high due to fatal congenital defects and secondary infections that cause enteritis, pneumonia, and arthritis (McClurkin et al., 1979; McClurkin et al., 1984), in some situations 17–50% of PI calves may reach breeding age (Barber et al., 1985; Binkhorst et al., 1983; Houe, 1993). Persistently infected females of breeding age are not only a source of horizontal transfer of BVDV, but also will produce a PI calf themselves, which if it survives, creates a familial clustering of PI animals (Houe et al., 1995; McClurkin et al., 1979).

The consequence of introducing a PI animal into a beef herd (confined breeding and calving seasons) depends on the timing of the introduction relative to the breeding season and the subsequent immunologic status of the herd during early gestation (Table 14.1). A likely scenario for a BVDV-exposed herd is to experience an initial peak of disease followed by low-level chronic reproductive losses in subsequent months and years. If a PI animal enters the herd either by birth or by purchase near the start of the breeding season, a high percentage of the herd may not be immunologically protected to the degree necessary to prevent viremia, conception failure, abortion, or fetal infection. If the PI animal is in contact with the breeding herd for a long enough period of time, the majority of the herd should become infected and seroconvert. Seropositive animals are less likely to have conception failures, abortions, or infected fetuses compared to seronegative animals. If no intervention is applied to the herd, the number of susceptible females the following year should be greatly decreased and the number of abortions and

Table 14.1. Various outcomes of bovine viral diarrhea virus (BVDV) infection depending on transmission and host.

Timing and Route of Transmission	Infected Animal	Outcome
Postnatal horizontal transmission	Seropositive nonpregnant animal	<ul style="list-style-type: none"> • Mild or subclinical disease
	Susceptible nonpregnant animal	<ul style="list-style-type: none"> • Mild or subclinical disease • Moderate to severe clinical disease with virulent strains
	Susceptible breeding animal	<ul style="list-style-type: none"> • Conception failure • Conception success
	Susceptible pregnant animal	<ul style="list-style-type: none"> • Fetal infection
Gestational vertical transmission	Fetus of persistently infected dam	<ul style="list-style-type: none"> • Fetal infection <ul style="list-style-type: none"> ◦ Embryo loss/abortion ◦ Immunotolerant, persistently infected fetus/calf
	Fetus of susceptible dam (d 0 to d 90–125 gestation) — cytopathic biotype	<ul style="list-style-type: none"> • Fetal infection <ul style="list-style-type: none"> ◦ Embryo loss/abortion
	Fetus of susceptible dam (d 0 to d 90–125 gestation) — noncytopathic biotype	<ul style="list-style-type: none"> • Fetal infection <ul style="list-style-type: none"> ◦ Embryo loss/abortion ◦ Immunotolerant, persistently infected fetus/calf ◦ Fetal malformations (d 100–150): cerebellar hypoplasia, hypotrichosis, brachygnathism, depleted lymph nodes/tissue, etc.
	Fetus of susceptible pregnant animal with immune-competent fetus (>90–125 d)	<ul style="list-style-type: none"> • Fetal infection <ul style="list-style-type: none"> ◦ Abortion ◦ Fetal malformations ◦ Normal fetus/calf
	Fetus of seropositive pregnant dam (seropositive following natural infection)	<ul style="list-style-type: none"> • Fetal protection from infection • Fetal infection ?
	Fetus of seropositive pregnant dam (seropositive following vaccination)	<ul style="list-style-type: none"> • Fetal protection from infection • Fetal infection <ul style="list-style-type: none"> ◦ Embryo loss/abortion ? ◦ Immunotolerant, persistently infected fetus/calf ◦ Normal fetus/calf

infected fetuses (both PI and immunocompetent) should decrease. Thus, even in the absence of vaccination and culling, the number of PI animals and BVDV infections in a closed herd may be self-limiting over time (Houe, 1995). A model developed by Cherry et. al. (1998) indicates that in continuous calving situations, the proportion of PI animals in a dairy herd will reach an equilibrium of about

0.9–1.2% in closed herds with no BVDV control procedures (Cherry et al., 1998). This model does not hold true if the herd is not closed (replacement females and bulls are added). Prevalence of PI animals and the resulting problems would not be expected to reach equilibrium in BVDV-infected herds with no control measures that are purchasing heifers, particularly bred heifers.

Estimates of the prevalence of PI animals in the general cattle population range between 0.13% and 2.0% (Bolin et al., 1985; Houe et al., 1995; Howard et al., 1986; Wittum et al., 2001). Differences in reported prevalence may be due to the population tested, the country/continent where the population was located, management system in effect and/or the diagnostic tests utilized. Persistent infection has a clustered distribution, which means a few herds may contain several cattle but most herds contain only non-PI cattle (Bolin, 1990). Clustering of multiple PI animals in a herd is primarily due to exposure of numerous susceptible dams to a PI or transiently infected source of noncytopathic BVDV prior to day 125 of gestation, but can also be due to surviving PI offspring of a PI dam.

DIAGNOSTIC TESTS FOR THE DETECTION OF PI ANIMALS

Because the PI animal is an important reservoir and transmitter of BVDV, control programs must first identify and remove these animals from the breeding herd. Because of vertical transmission of the virus from viremic dams to their fetuses, PI animals should be removed prior to the start of the breeding season in beef herds with a controlled breeding season. In dairy herds, the PI animals must be removed as soon as possible from direct or indirect contact with the breeding herd. In order to find and remove PI beef cattle prior to the start of the breeding season, all calves, all replacement heifers, all bulls, and all nonpregnant dams without calves must be tested for PI status (Kelling et al., 2000). Nonpregnant cows may lack calves prior to the start of the breeding season due to not becoming pregnant, aborting, or calf mortality. Any female that is still pregnant at the time the herd is tested should be isolated from the breeding herd and kept isolated until her calf is tested and found to be negative. Calves persistently infected with BVDV can be identified by testing strategies that utilize virus isolation from whole blood (buffy coats) or serum, immunohistochemistry (IHC) staining of viral antigen in skin biopsies, antigen-capture enzyme-linked immunosorbent assay (ELISA) from skin biopsies, and polymerase chain reaction (PCR) methods from whole blood or serum (Dubovi, 1996)

VIRUS ISOLATION

Persistently infected animals produce an exceptionally large number of BVDV particles that can be isolated from virtually any tissue sample, including blood, serum, spleen, liver, and lymphoid tissue

(Ellis et al., 1995; Straver et al., 1983). Virus isolation is considered to be very specific for BVDV infection; however, colostral antibodies may temporarily reduce the amount of free virus in the serum of young calves, thus making it less sensitive to isolate virus from the blood or serum of young calves (Kelling, et al., 1990; Palfi et al., 1993; Brock et al., 1998). However, when the maternal antibodies have disappeared, BVDV can be isolated easily from PI calves. BVDV could be isolated by 6 weeks of age in colostrum-fed PI calves in one study (Brock et al., 1998), and by 8 weeks of age in colostrum-fed PI calves in another study (Palfi et al., 1993). Although it is rare, some PI calves develop neutralizing antibody and clear the persistent BVDV strain from their serum. However, in these animals it is still possible to isolate virus from leukocytes (Brock et al., 1998). Virus isolation methods are labor-intensive and take several days to complete. An additional shortcoming is that virus isolation may not differentiate between transiently infected animals and PI animals, unless positive cattle are re tested and remain positive at a later date (i.e., 3 weeks later).

IMMUNOHISTOCHEMISTRY

An IHC test for BVDV infection using skin biopsy samples, such as ear notches, is available that differentiates between PI animals and transient BVDV infections (Njaa et al., 2000). Transiently infected animals may have internal organ tissue samples that are IHC-positive. However, when skin samples were evaluated, transiently infected animals either had no staining, or staining was confined to the epidermal keratinocytes and follicular ostia, in contrast to PI cattle with antigen-positive staining cells in all layers of the epidermis, all levels of hair follicles, and the hair bulb (Njaa et al., 2000). This test is suitable for herd screening because samples can be taken from cattle of any age, sample collection is simple, the samples are stable for transport and handling, it is not affected by the presence of passive antibodies and the test is both sensitive and specific for PI cattle (Ellis et al., 1995; Baszler et al., 1995; Njaa et al., 2000). In addition, the use of modified live BVDV vaccine does not appear to produce false positive IHC reactions on skin biopsies when testing for PI animals (DuBoise et al., 2000).

POLYMERASE CHAIN REACTION

Polymerase chain reaction testing for BVDV infection is more rapid than virus isolation and can also detect virus in antigen-antibody complexes (Brock et al., 1998). Polymerase chain reaction tests are

sensitive and have been shown to differentiate between BVDV genotypes. However, a single BVDV-positive blood sample tested by PCR does not allow the diagnostician to differentiate between viremia from a postnatal acquired infection and that from being a PI animal. Because PCR tests can identify minute amounts of virus, this test can be used in pooled samples of blood or milk in surveillance programs.

SEROLOGY

Although PI cattle are usually seronegative to BVDV, an immune response can be elicited to a heterologous strain (Brock et al., 1998). Presumably, this response can follow either natural or vaccine exposure. In addition, some cattle in both vaccinated and unvaccinated herds are seronegative, making serology alone unsuitable for identification of PI animals (Bolin et al., 1985; Houe et al., 1995; Wittum et al., 2001).

DIAGNOSTIC TESTING STRATEGIES TO IDENTIFY PI CALVES

If a herd has had confirmed PI calves, or if the history strongly suggests the presence of PI calves, the a priori assessment of PI prevalence is fairly high, making the predictive value of a positive test high enough so one can conclude that the animal is persistently infected and that PI animals are present in the herd. Consequently, a second confirmatory test may not be needed. In contrast, if there is no a priori evidence of PI prevalence greater than 0.3–0.5%, the predictive value of a positive test is low and a different confirmatory test may be advisable before making conclusions about the individual animal or the herd (Table 14.2). When a calf is identified as PI, it

should be euthanized or removed for slaughter and the dam should be tested. Most dams of PI calves are not PI themselves, and if confirmed as non-PI, can reenter the breeding herd because naturally acquired immunity is considered to prevent future fetal infections (Orban et al., 1983). Dams identified as PI, however, should be sold to slaughter immediately.

In most whole-herd testing situations, IHC testing of skin samples is currently the test of choice because it can be accurately performed on animals of any age and a single sample is all that is usually sufficient. The use of virus isolation or PCR to identify BVDV-infected cattle requires a second test 3 weeks following any positive results to differentiate between transient viremia and PI with BVDV.

IDENTIFICATION OF DAMS CARRYING A PI FETUS

Purchase of non-PI dams pregnant with PI fetuses (PI-carriers) is a potential source of BVDV spread between herds. Any pregnant heifer or cow that is purchased should not come in contact with other pregnant cattle unless they have been tested and confirmed not to be PI. In addition, because the status of the fetus is unknown, the calf must be tested soon after birth and prior to the breeding season to prevent contact with pregnant cattle in the first 210 days of gestation. Because it would be advantageous to identify PI fetuses prior to purchase or entry of the dam into a herd, other strategies to determine the PI status of the fetus are being investigated.

Unvaccinated non-PI dams carrying PI fetuses are seropositive and virus-negative, but they have markedly higher titers of antibodies to BVDV compared to dams carrying healthy fetuses (Lindberg et al., 2001). Optical density (OD) of an indirect ELISA technique has a strong positive correlation to

Table 14.2. Possible tests to identify persistently infected (PI) cattle.

Initial Test	Confirmatory Test
Immunohistochemistry (IHC) of skin biopsy	Virus isolation (VI) from serum or whole blood or Polymerase chain reaction (PCR) from serum or whole blood
VI-positive from two whole blood samples taken 3 weeks apart	IHC of skin biopsy
PCR-positive from two whole blood samples taken 3 weeks apart	IHC of skin biopsy

virus neutralization titer, and for unvaccinated seropositive dams carrying normal calves, the average OD value stays the same irrespective of when in pregnancy the sample is taken (Lindberg et al., 2001). In contrast, unvaccinated dams that carry PI fetuses have an increasing antibody titer from early pregnancy (when they become infected) through the last month of gestation, resulting in significantly higher OD by the 7th month of gestation compared to dams carrying normal calves (Lindberg, 2001). The sensitivity of this method to identify dams carrying PI calves ranged from 0.79–0.96 and the specificity ranged from 0.37–0.70, depending on the test cutoff (Lindberg et al., 2001). Because of the relatively high sensitivity, relatively low specificity, and low prevalence of dams carrying PI fetuses, the negative predictive value will be high and the positive predictive value will be poor. Therefore, a negative test should be considered good evidence for the absence of a PI fetus, but a positive test would not be a particularly strong indication for the presence of a PI fetus in tested unvaccinated dams in herds with endemic BVDV.

Another method that has been investigated in an effort to identify dams carrying a PI fetus is the collection of fetal fluids and their evaluation for the presence of BVDV (Callan et al., 2002). Callan et al. (2002) were able to use abdominal ultrasound to guide a spinal needle introduced through a surgically prepared area of the right flank into the pregnant uterus to collect a sample of fetal fluid. Virus isolation performed on the fetal fluid samples correctly identified 1/1 BVDV-infected and 168/168 noninfected fetuses. In addition to the technical expertise required, this method may have limited application in most herds because 8% (14/169) of tested dams aborted or delivered premature calves within 3 weeks of fetal fluid collection that may have been related to the sample collection (Callan et al., 2002).

MONITORING HERDS FOR BVDV PI RISK

The cost of initiating a BVDV PI whole-herd screening protocol on a farm or ranch is significant. Because of the low prevalence of herds with at least one PI animal, veterinary practitioners may not be economically justified to initiate whole-herd screening protocols to find PI BVDV beef cattle for herds at low risk for the presence of PI cattle or herds that cannot gain significant market price advantage for selling groups of cattle that have been tested and determined to be free of PI individuals (Wittum et al.,

2001; Larson et al., 2002). However, if ranch history raises a suspicion of BVDV PI cattle being present in the herd, or if significant marketing advantages exist, a protocol to screen the herd can be defended based on its likelihood to improve or protect economic return (Larson et al., 2002). Several strategies can be employed to monitor herds for their risk of having PI cattle present. The interpretation of results from these strategies would be different if the goal were to monitor for the presence of BVDV rather than PI animals. If complete eradication of BVDV is desired, the effort and cost of monitoring is much greater than for monitoring for the presence of PI cattle.

USE OF PRODUCTION RECORDS AND LABORATORY EVALUATION OF MORIBUND AND DEAD CALVES

The minimal level of surveillance for every herd should include monitoring of herd fertility (early breeding season pregnancy proportion, pregnancy per insemination proportion, and total pregnancy proportion), neonatal calf morbidity and mortality proportions, and weaning proportions. Because of the negative effect of the presence of PI calves in a breeding herd on measures of reproductive efficiency, the presence of physical abnormalities at birth, and calf survivability to weaning, an unacceptable level of these symptoms increases the risk that BVDV is a problem in the herd and increases the likelihood that whole-herd screening for PI cattle will be economically rewarding (Houe and Meyling, 1991). Although, in many situations, pregnancy rate drops significantly at the time of conception of the oldest PI animal, and about 6 months later calf mortality increases; using production records alone lacks sensitivity for identifying herds with PI animals because the clinical indications of PI presence may be less noticeable in some outbreaks (Houe and Meyling, 1991). The clinical signs and time sequence following introduction of BVDV infection into different herds varies considerably due to the different proportions of seronegative animals in the critical period of pregnancy and different virulence among BVDV strains (Houe, 1995).

In addition to monitoring production records, minimal surveillance should include the necropsy examination of as many aborted fetuses, stillborn calves, and calves that die preweaning as possible, with whole blood submitted for determination of BVD viremia and serum submitted for serologic evidence of infection. In addition, moribund calves from clusters of pneumonia, neonatal scours, or sep-

ticemia outbreaks that are not easily explained by sanitation or other problems should also be tested for BVDV exposure and PI status. If most perinatal and preweaning mortalities are examined for BVDV antigen via IHC and found to be negative, it is not likely that PI animals are present in the herd. The presence of PI animals in the herd will be established by a single confirmed IHC-positive skin sample. The presence of PI animals is not ruled out and may be considered likely if few moribund or dead cattle are tested and found to be IHC-negative, but other tests indicate the presence of viremia or serology indicates recent BVDV infection and the possibility of PI animals being in contact with the moribund or dead sample animals (Table 14.3).

The advantage of utilizing production measures and necropsies to determine whether herds have either a high or low risk for the presence of BVDV PI animals is that minimal expense is involved and these management tactics are inclusive for the monitoring of other disease and production problems. This level of monitoring is probably appropriate in herds with no evidence for the presence of PI animals and that are at low risk of PI introduction (Larson et al., 2002). The disadvantage is that at least one PI animal is allowed into the herd before production losses are identified, and production losses will continue for at least 1 year after intervention is initiated.

USE OF POOLED SAMPLES OF WHOLE BLOOD FOR PCR TESTING

Herd monitoring for the introduction of PI animals can also be accomplished with pooled whole blood samples for PCR testing. By pooling samples, the expense of screening herds with a low prevalence of PI animals is minimized. Polymerase chain reaction is well suited to pooled-sample testing for the presence of BVDV PI animals because it is sensitive enough to detect minute amounts of virus. A single PI animal was detectable in pools of 200–250 negative samples (Muñoz-Zanzi et al., 2000). Animals contributing to negative samples are all assumed to be non-PI, whereas positive pools may contain samples from PI animals or transiently viremic animals. If the initial pool is PCR-positive, it must be split and retested to differentiate viremic and non-viremic animals. After the viremic animals are identified, they must be classified as transiently infected or PI with either a subsequent PCR or virus isolation test in 3 weeks or via IHC of a skin sample.

The best size of the initial pool is determined by the balance between the cost savings of having large

Table 14.3. Diagnostic test results of clinically ill animals with BVDV infection.

BVDV infection status	IHC ^a	Serology	Viremia (PCR ^b /VI ^c)
Transiently infected	–	–	+
Convalescent	–	+	–
Persistently infected (PI)	+	+/-	+

^aImmunohistochemistry.

^bPolymerase chain reaction.

^cVirus isolation.

numbers of individuals represented in negative pools and few individuals represented in positive pools that require further diagnostics. If pool size is too large, there is an increased chance that any single pool will test positive, requiring additional testing to identify the few truly viremic individuals in the pool. If the samples are grouped in unnecessarily small pools, the cost benefit of pooling samples is lost to the large number of negative pools tested for each positive pool identified (Muñoz-Zanzi et al., 2000). Muñoz-Zanzi et al. (2000) developed a simulation model to determine that the economically optimum sample size depends on prevalence of true positives in the population. For a PI prevalence of 0.5–1.0%, the optimum number of samples in an initial pool is 20–30, using a described repooling strategy for test-positive initial pools (Muñoz-Zanzi et al., 2000). As prevalence increases, the least-cost initial pool size decreases (Muñoz-Zanzi et al., 2000).

If whole blood samples are collected for pooled PCR from all suckling calves prior to the start of the breeding season, PI cattle can be identified and removed before contact with pregnant females, thereby eliminating the opportunity for a PI animal within the herd to cause reproductive failure and to create more PI animals in the next calf crop. Screening for PI animals at a later time, such as weaning, is discouraged. If samples are taken at weaning, although PI cattle can be removed from the herd, those continuously viremic animals were in contact with pregnant females throughout much of gestation and can cause reproduction and production losses, including the creation of PI cattle in the next calf crop.

USE OF SEROLOGIC EVALUATION OF SENTINEL ANIMALS

Herd surveillance of dairy herds has been described using sentinel animals. Pillars and Grooms (2002)

found that serologic evaluation of unvaccinated 6- to 12-month-old heifers for the presence of a high serum neutralizing titer had a sensitivity of 66% and specificity of 100% for detecting herds that have PI cattle present when reference strains from both genotypes 1 and 2 were used. Herds that were identified as containing PI animals could then utilize other diagnostic tests to identify individual PI animals for removal. In countries that have BVDV control programs that do not allow the use of vaccination, a similar strategy to identify herds with PI cattle has been investigated. This strategy is based on the fact that in unvaccinated herds, there are significantly more antibody-positive animals, especially in young stock, in herds with PI animals than in herds without PI animals (Houe, 1992).

However, because of the variable percentage of antibody-positive animals in herds without PI animals, it was not possible to predict the presence of PI animals in dairy herds in The Netherlands using this method (Zimmer et al., 2002). Because this monitoring occurs when potential PI animals are at least 6 months old, a positive herd test would indicate that the herd has been exposed to at least one PI animal for at least 6 months, reproductive losses and the presence of PI calves in the next calf crop would be expected. When utilizing serology strategies for BVDV monitoring or diagnostics, it is important to use reference strains from both genotypes 1 and 2 because it is not unusual for BVDV-exposed cattle to have a low titer against one genotype and a higher titer against the other.

USE OF ANNUAL WHOLE-HERD TESTING

Certain high biosecurity herds, such as herds selling or developing replacement breeding animals, may elect to undergo a high level of surveillance even in the absence of evidence that PI animals are present. This high level of biosecurity may be important to their marketing plan or may indicate a high value placed on avoiding the small, but real, risk of introducing BVDV virus into the herd with subsequent negative reproductive, health, and marketing consequences. The first year that a beef herd adopts this strategy, all suckling calves, all females that were bred that failed to present a calf on test day, all replacement heifers, and all bulls should be tested. If any calf is confirmed as a PI animal, his dam should be tested as well. In subsequent years, only suckling calves and any purchased animals need to be tested. If pregnant animals are purchased, the dam should be tested prior to or at arrival and the calf should be tested immediately after birth. In beef herds with a

confined breeding season, this testing must occur before the start of the breeding season to ensure that no PI animals are in contact with pregnant females during gestation. Heifer development operations should test every heifer prior to or at arrival at the facility. Dairy operations should test every calf that stays on the same farm as the breeding herd or is cared for by breeding herd employees, or if any equipment used on the calf-rearing farm is used on the breeding herd farm.

Following the identification and removal of PI animals from a herd, testing of all suckling calves should be done for one or more breeding seasons to ensure the complete accounting for PI animals. Because no test or testing strategy is perfectly sensitive, and because risk factors involved in the initial introduction may still be present, a vigilant monitoring system is wise until a high confidence for PI-free status is achieved.

OTHER POTENTIAL SOURCES OF BVDV

Male PI calves will occasionally be selected for use as breeding bulls. The amount of BVDV excreted in the semen of persistently infected bulls is very high (10^4 – 10^6 TCID₅₀/ml) (Revell et al., 1988). When BVDV is infused into the uterus at the time of breeding, seronegative cattle exhibit a significant reduction in conception rate, but seropositive animals may not be adversely affected (Whitmore et al., 1981). BVDV-contaminated semen is an efficient horizontal transmitter of disease from bull to cow (Paton, et al., 1989). If PI bulls are used for natural service, the cows may conceive when immunity has developed, resulting in the birth of normal (non-PI) calves (Barber et al., 1985; McClurkin et al., 1979). If PI bulls are used for AI, all or most seronegative females bred with the semen will become infected with BVDV although most will not produce a PI calf (Meyling and Jensen, 1988).

Although some evidence exists for BVDV to cause latent infections, particularly in gonads and accessory sex glands (Kirkland, et al., 1991; Voges et al., 1998), recrudescence and excretion in immune-competent animals has not been shown to date to be involved in the epidemiology of the disease (Brownlie, 1990). And although PI bulls will shed BVDV in semen for prolonged periods of time, virus excretion in semen from transiently infected bulls was confined to days 10–14 post-experimental-infection, and the virus titer in semen of transiently infected bulls was much lower than for PI bulls (Paton et al., 1989).

EMBRYO TRANSFER

Embryo transfer is a potential route of transmission of BVDV. If the embryo recipient is PI, vertical transmission to the transferred embryo will occur causing embryonic loss or the creation of a PI fetus. Although there is no evidence to suggest that BVDV is present inside the embryos of viremic females, the virus can be present on the intact zona pellucida of PI and transiently infected females, and the virus is present at high levels in the uterine environment of PI donors (Singh et al., 1982). Established washing procedures will remove contaminating virus, but if these procedures are not followed, BVDV from the collection fluids or virus present on the zona pellucida can be horizontally transferred to a susceptible recipient cow (Singh, 1982; Singh et al., 1985). Vertical transmission from the recipient cow to the fetus can occur, resulting in embryonic/fetal death or the birth of a PI calf (Brock et al., 1991). BVDV infection of the recipient cow and fetus can also occur when both the donor and recipient are free of BVDV if BVDV-contaminated fetal serum is used in the embryo transfer process or if contaminated liquid nitrogen is in direct contact with embryos (Singh et al., 1985; Bielanski et al., 2000).

OTHER UNGULATE SPECIES (DOMESTIC AND WILDLIFE)

Other ungulate species may be potential sources of BVDV to susceptible cattle herds. Transmission of BVDV between sheep and cattle has been demonstrated, but the importance of this transmission has not been established (Carlsson and Belak, 1987). BVDV has also been isolated from pigs, but again, the importance of pigs as a source of the virus to susceptible herds is not established (Liess and Moenning, 1990; Terpstra and Wensvoort, 1988). Deer seropositive to BVDV have been identified in North America and Europe (Davidson and Crow, 1983; Nielsen et al., 2000; Frolich et al., 2002). However, the existence of PI deer has not been demonstrated, and cattle are assumed to be the source of BVDV infection for free-ranging ruminants.

FOMITES

Fomites may serve in the transmission of BVDV from PI cattle to susceptible animals. A 19-gauge needle was able to infect susceptible cattle with BVDV when used IV within 3 minutes of drawing blood from a PI animal (Gunn, 1993). Nose tongs were able to infect susceptible cattle with BVDV when used for 90 seconds within 3 minutes of being used in a PI animal (Gunn, 1993).

No evidence has been presented that insects are a source of BVDV transmission in field outbreaks (Table 14.4). However, a role is possible: BVDV was isolated from nonbiting flies (*Musca autumnalis*) collected from the face of a PI animal, and experimental BVDV transmission between a PI animal and susceptible animals occurred when 50 biting flies fed on the PI animal for 5 minutes and 15 minutes later fed on susceptible animals (Gunn, 1993; Tarry et al., 1991).

VACCINATION TO CONTROL BVDV-INDUCED DISEASE AND PRODUCTION LOSSES

In addition to removal of PI reservoirs, BVDV transmission to and within the herd can be reduced with an appropriate vaccination program. Information from serological data and limited field trials can be used to make empirical recommendations regarding what constitutes an effective vaccination program to limit postnatal and gestational BVDV transmission.

IN VITRO EVIDENCE OF VACCINE EFFICACY

Although there were large variations in the vaccine-induced virus neutralizing antibody titers of individual colostrum-deprived calves vaccinated with two doses (21-day separation between doses) of an inactivated BVDV vaccine (Hamers et al., 2002) or with a modified live, temperature-sensitive BVDV vaccine (Hamers et al. 2000b), sera from all animals were capable of neutralizing a wide range of antigenically diverse European and American isolates of BVDV, including genotypes 1 and 2. In another study, administration of a single dose of a modified live BVDV vaccine in seronegative cows induced antibodies that were able to cross-neutralize 12 antigenically diverse strains of BVDV (Cortese et al., 1998c) and were detectable for at least 18 months.

COLOSTRAL IMMUNITY AND VACCINATION OF YOUNG CALVES

Adequate intake of colostrum from BVDV seropositive dams provides protection from clinical disease in young calves (Cortese et al., 1998b; Ridpath et al., 2003). Vaccination of young calves has also been demonstrated to reduce clinical disease and mortality following experimental challenge (Cortese et al., 1998b). Calves that did or did not receive colostrum antibodies and were vaccinated at 10–14 days of age with a single dose of modified live vaccine (MLV) containing type-1 BVDV were protected from clinical disease when experimentally challenged 21 days postvaccination with a virulent type-2 BVDV. In

Table 14.4. Sources of bovine viral diarrhea virus and mode of transmission to susceptible cattle.

Sources of BVDV for Transmission to Susceptible Cattle with Convincing Evidence to Support Their Role in the Epidemiology of BVD

Source of BVDV	Target Animal	Mode of Transmission
Cattle persistently infected with and immunotolerant to BVDV	Postnatal cattle	Direct horizontal oronasal contact with viral-contaminated secretions and excretions
Transiently infected cattle	Postnatal cattle	Direct horizontal oronasal contact with viral-contaminated secretions and excretions
Dam persistently infected with and immunotolerant to BVDV	Fetus	Vertical transmission across placenta from maternal viremia
Transiently infected dam	Fetus	Vertical transmission across placenta from maternal viremia

Sources of BVDV for Transmission to Susceptible Cattle with Experimental or Serologic Evidence to Support a Potential Role in the Epidemiology of BVD

Source of BVDV	Target Animal	Mode of Transmission
Domestic farm animals: sheep, pigs, goats	Postnatal cattle	Direct horizontal oronasal contact with viral-contaminated secretions and excretions
Wildlife: deer, elk	Postnatal cattle	Direct horizontal oronasal contact with viral-contaminated secretions and excretions
Fomites: palpation sleeves, nose tongs, injection needles	Postnatal cattle	Indirect horizontal oronasal contact with viral-contaminated secretions and excretions
Flies	Postnatal cattle	Blood meals

contrast, calves that did not receive colostral antibodies to BVDV and did not receive the MLV vaccine suffered severe clinical disease and required euthanasia (Cortese et al., 1998b). Clinical scores, indicating severity of illness, were not significantly different between seropositive-vaccinated and seropositive-unvaccinated calves after experimental viral challenge in this trial (Cortese et al., 1998b). Most of the vaccinated calves that were seronegative prior to vaccination did not have measurable serum antibody response 21 days following vaccination at the time of experimental challenge, even though these calves were protected from clinical disease (Cortese et al., 1998b). Similarly, Ridpath et al. (2003) demonstrated that an active protective response, not correlated to serum-neutralizing antibodies, was mounted in young calves in the presence of colostral-derived passive immunity. Thus serum antibody titers may represent an inadequate

measure of protection against disease (Cortese et al., 1998b; Ridpath et al., 2003).

ABILITY OF VACCINES TO PROVIDE FETAL PROTECTION

Using vaccination strategies to prevent or reduce clinical disease caused by BVDV is important in the management of bovine respiratory disease in feedlot and other confined cattle situations. However, when controlling BVD in cowherds, controlling clinical disease is minimally important compared to preventing fetal infection that results in embryonic/fetal loss or creation of PI animals. Fetal protection is immunologically more difficult than protection from clinical disease; however, the majority of vaccines are licensed based on their ability to reduce clinical signs in acute infection, not to reduce reproductive loss or fetal infection. Fetal infection can be prevented if the immune system of an exposed herd is

primed so that it can effectively neutralize circulating virus before it has a chance to cross the placenta and cause fetal infection. Evidence from earlier as well as recently reported trials indicates that maternal vaccination provides some protection of the fetus, although protection does not extend to 100% of fetuses (Kelling et al., 2000). In fact, efficacy of maternal vaccination to provide fetal protection has been reported to range from 25–100% for inactivated vaccines (Meyling et al., 1987; Harkness et al., 1987; Brownlie et al., 1995) and from 58 to 88% for modified live vaccines (Cortese et al., 1998a; Brock and Cortese, 2001). The presence of measurable levels of BVDV antibody in dams following vaccination appears to provide fetal protection and is important in a planned vaccination program for BVDV control. It should be realized, however, that a sufficient amount of virus is able to escape neutralization inactivation by circulating antibodies in some dams to cause transplacental infection, abortion, and the development of persistent fetal infection. Thus, vaccination programs by themselves are inadequate in controlling BVDV (Cortese et al., 1998a; Brock and Cortese, 2001; Brownlie et al., 1995).

CONTROL PROGRAMS TO LIMIT LOSSES DUE TO BVDV

BEEF CATTLE

The primary goals of BVDV control in breeding herds are to prevent fetal infection in order to eliminate BVDV-associated reproductive losses (thereby preventing the birth of PI calves) and to reduce losses from transient BVDV infections (Harkness, 1987). Cattle that have been infected with BVDV after birth and have recovered are considered to be protected from clinical disease following subsequent exposure to the virus even if they are seronegative (Ridpath et al., 2003). Animals that are seropositive in response to natural exposure are also considered to be protected from future transmission of the virus to a fetus. An immunocompetent animal that gives birth to a persistently infected animal will develop high antibody titers and is likely to eliminate future BVDV infections rather than pass them to the fetus. Thus, an immunocompetent dam (non-PI) could have, at most, one PI calf.

While vaccination does provide some protection from fetal infection, the herd level protection is not equal to that generated by natural exposure. As a result, BVDV control is generally achieved by a combination of removal of PI cattle, vaccination, and a

biosecurity system that prevents the introduction of PI animals into the herd and minimizes the contact with potentially viremic cattle or wildlife (Kelling, 1996).

Removal of PI animals

Herds should be routinely monitored to determine the presence of PI animals. If the presence of PI cattle is confirmed or strongly suspected, a whole-herd screening protocol, most likely utilizing IHC of skin samples or PCR as described earlier, should be undertaken to identify and remove PI individuals. A second whole-herd screening the following year may be advisable in some herds where risk of continued fence-line or other exposure to PI animals is high.

Biosecurity to prevent herd exposure to PI animals

Biosecurity to prevent herd exposure to PI or transiently infected animals is important, especially after the removal of PI cattle, because with the removal of PI animal (BVDV) shedders, the numbers of naturally exposed seropositive animals in a herd decreases (Kelling, 1996). All replacement heifers and bulls that enter the breeding herd, whether raised or purchased, should be tested prior to the start of breeding to ensure that they are not PI animals. If a pregnant animal is purchased, it should be segregated from the breeding herd until both the dam and the calf are confirmed to be non-PI. Fence-line contact with neighboring cattle should be managed so that stocker cattle are not adjacent to the breeding herd during early gestation, and other cowherds are not adjacent unless they also have a strict biosecurity and vaccination program in place.

Vaccination as a component of biosecurity

Biosecurity also involves application of a vaccination protocol to reduce the risk of fetal infection in the event of cowherd exposure to a viremic and virus-shedding animal. Modified live vaccines are believed to stimulate more complete protection against transplacental infection (Kelling, 1996). For that reason, one recommendation is to vaccinate unstressed, healthy heifers with MLV vaccines. Vaccine administration should be timed so that a protective immune response coincides with the first 4 months of gestation. This is done to maximize the potential for adequate immunity to protect against fetal infection and reproductive failure or the birth of PI calves. If heifers have not been previously vaccinated, the primary vaccination should be done

Table 14.5. Control program for BVDV in beef cow herds.

I. Monitor herd for risk of PI presence	ii. Use vaccines according to label to booster cowherd annually
a. If PI presence is confirmed or strongly suspected, a whole-herd screening to identify and remove PI individuals is undertaken	c. Biosecurity to prevent introduction of virus into herd
b. Monitoring options:	i. Test purchased bulls and heifers
i. Monitor production (reproduction efficiency, neonatal and postnatal death loss), monitor ill neonates for BVDV viremia, and necropsy (with laboratory submissions) as many abortions, stillbirths, and neonatal deaths as possible	ii. Test raised replacement heifers and bulls
ii. Use pooled samples of whole blood taken from suckling calves prior to breeding for PCR testing	iii. Monitor population for evidence of introduction of PI animals
iii. Use serologic evaluation of sentinel animals	d. Purchasing bred heifers
iv. Use annual whole-herd screening prior to breeding season (all suckling calves and purchased replacements)	i. If heifers are tested prior to purchase, still need to test calf after birth.
II. Biosecurity for herd against BVDV entrance	ii. If heifers are not tested prior to purchase, can test heifer prior to entry into herd and then test calf after birth, or isolate heifer until calf is born and test the calf (only need to test heifer if calf is positive).
a. Isolate herd during breeding/gestation from cattle of unknown BVD PI status (avoid fence-line contact with stocker cattle, neighbors' cattle, etc.)	iii. Percutaneous collection of fetal fluids for detection of PI calf has been described – not recommended at this time
b. Vaccinate to reduce virus circulation and to reduce production of PI calves in face of virus circulation	iv. Serology in late gestation to identify PI-carrying unvaccinated dams (Good negative predictive value, poor positive predictive value)
i. Use MLV vaccine two or more times between weaning and two months prior to first breeding as heifers	e. Fomites
	i. Maintain sanitation to prevent viral spread via nose tongs, injection needles, palpation sleeves, etc.

twice. The first dose should be given when the heifers are 6 months of age or older, and the second dose should be given 2 months before breeding. Beef cows should be revaccinated annually before breeding according to label directions (Kelling, 1996).

DAIRY CATTLE

The management of U.S. dairies is vastly different than that of beef cows; dairy calves are removed from their dams soon after birth and are not in contact with the breeding herd during gestation. Hence, calf transmission of BVDV to pregnant females is eliminated or greatly reduced in dairy herds as compared to beef herds. However, because many pregnant replacement females are purchased or raised off-site on dairies, the risk of introduction of a PI replacement animal or pregnant non-PI an-

imal with a PI fetus is greater in dairy operations than in beef herds. Biosecurity for dairy herds should include screening of potential replacement heifers prior to the start of their first breeding season, biosecurity of heifer and adult cows during gestation to prevent exposure to PI or transiently infected animals, and a vaccination program to provide some level of protection in the face of exposure to viremic animals.

STOCKER/FEEDLOT OPERATIONS

Because pregnancy is not a common or desirable component of stocker and feedlot operations, vertical transmission and reproductive losses due to BVDV are not a concern. However, BVDV viremia or seroconversion has been associated with respiratory disease outbreaks in feedlot situations (Martin et al., 1989; Fulton et al., 2000; Fulton et al., 2002).

Table 14.6. Control program for BVDV in dairy herds.

-
- I. Monitor herd for risk of PI presence
 - a. If PI presence is confirmed or strongly suspected, a whole-herd screening to identify and remove PI individuals is undertaken
 - b. Monitoring options:
 - i. Monitor production (reproduction efficiency, neonatal and postnatal death loss), monitor ill neonates for BVDV viremia, and necropsy (with laboratory submissions) as many abortions, stillbirths, and neonatal deaths as possible
 - ii. Use serologic evaluation of sentinel animals
 - iii. Test all heifer calves that may be saved as replacements (IHC of skin samples or PCR of pooled whole blood samples)
 - II. Biosecurity to prevent introduction of BVDV into herd
 - a. Test all heifer calves that are potential replacements if raising own replacements
 - b. Test purchased replacement heifers and bulls prior to or at delivery
 - c. Obtain replacement heifers from source that has a strict biosecurity program in place
 - d. Use vaccines according to label to booster cowherd
 - e. Monitor population for evidence of introduction of PI animals
 - III. Biosecurity for heifer raisers
 - a. Test all heifers prior to or at arrival at development facility/farm
 - b. Isolate heifers during breeding/gestation from cattle of unknown BVD PI status
 - c. Vaccinate with MLV product two or more times between six months of age and start of breeding
-

Persistently infected cattle are a primary source of BVDV transmission to in-contact susceptible cattle during marketing, trucking, and while in feeding pens and pastures. Vaccination is currently the primary control intervention for BVDV in stocker and feedlot operations. Screening cattle for the presence of PI individuals prior to purchase or at arrival has not been adequately evaluated for economic return. The economic return will depend on the prevalence of PI cattle, the sensitivity and specificity of the test used, and the economic cost of the disease to the operation.

Table 14.7. Control program for BVDV in stocker/feedlot operations.

-
- I. Vaccination of incoming cattle with MLV product
 - II. Screening incoming cattle for BVDV PI animals
 - a. Low prevalence causes even a test with high specificity to have more false positive than true positives
 - b. Testing should be done prior to cattle being commingled and trucked. Transmission and infection likely to occur during stress and crowding of commingling and trucking.
 - III. Purchasing PI-free certified cattle
 - a. All cattle in a group are test negative (IHC of skin sample or pooled PCR of whole blood)
 - b. Economic benefit is determined by multiplying the cost of having a PI feeder calf present (increased pen morbidity, mortality, treatment failure, and performance) by the expected prevalence for similar cattle.
 - IV. Purchasing PI-low risk cattle
 - a. All cattle in the group originate from farm(s) with a complete vaccination program and BVDV PI screening protocol
 - V. Purchasing cattle of unknown PI risk
 - a. Cost of unknown status is determined by multiplying the cost of having a PI calf present by the expected prevalence for similar cattle
 - b. Cost of unknown PI risk is added to other costs for break-even calculation
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Conclusions and Future Research

Julia F. Ridpath and Sagar M. Goyal

PROGRESS IN BVDV RESEARCH

Since the first publication on bovine viral diarrhea virus (BVDV) 60 years ago (Olafson et al., 1946), significant progress has been made in elucidating the pathogenesis, transmission, diagnosis, and molecular virology of this virus. We have come to understand that the pathogenesis of BVDV is complicated and that it produces a multifaceted disease (Bolin et al., 1985; Brownlie et al., 1984; Corapi et al., 1989). Monoclonal antibodies (Mabs) have been developed (Moennig et al., 1987) and the genome of BVDV has been completely sequenced (Collett et al., 1988). Antigenic and genomic diversity has been found to be a hallmark of BVDV, making the control of this virus difficult. In addition to different biotypes (Gillespie et al., 1960), different genotypes and subgenotypes have now been identified (Pellerin et al., 1994; Ridpath et al., 1994). Biotype differences in cytopathology in culture are associated with the production of a nonstructural protein (NS3 or P80) in the cytopathic strains (Meyers et al., 1991). Cytopathic effect observed in vitro (i.e., cytopathic biotype) does not correlate with virulence in vivo, because all highly virulent viruses belong to the noncytopathic biotype (Ridpath et al., 2000). Although hemorrhagic syndrome has been associated only with viruses from the BVDV 2 genotype, variation in virulence exists in both the BVDV 1 and BVDV 2 genotype (Evermann and Ridpath, 2002; Fulton et al., 2000; Fulton et al., 2002; Liebler-Tenorio et al., 2003).

Numerous studies have been undertaken to elucidate the mechanism of development of persistently infected (PI) animals and how to control them (Harding et al., 2002; Ohmann, 1982; Ohmann et al., 1982; Van Campen and Woodard, 1997). It is widely accepted that PI animals are a major source of virus on the farm and in feedlots. Although viral

shed is limited and normally confined to a period of less than 7 days, transiently infected animals can also be a source of virus to herdmates. Both acutely and persistently infected bulls can shed virus in their semen with a minimal effect on the traditional measures of semen quality (Givens et al., 2003; Kirkland et al., 1991; Kommisrud et al., 1996; Revell et al., 1988). The presence of BVDV in semen leads to virus transmission by the reproductive route, resulting in abortions, early embryonic death, congenital infections and PI animals (Givens et al., 2003; Kirkland et al., 1991; Kommisrud et al., 1996; Kirkland et al., 1997; Meyling and Jensen, 1988; Paton et al., 1990; Schlafer et al., 1990).

Major advances have been made in detecting BVDV infection, including the use of monoclonal antibody- and polymerase chain reaction-based tests. Although BVDV may be spread by animals that are either persistently or acutely infected, the main emphasis until now has been on the detection of PI animals. This is because the removal of PI animals is considered to be integral to an effective control strategy. Eradication programs in Sweden, Finland, and Denmark rely on nonvaccination of cattle so that positive animals can be identified and removed easily (Greiser-Wilke et al., 2003). These programs are reported to be successful in reducing BVDV-positive herds. In Germany, vaccination with killed vaccine followed by a booster dose of modified live vaccine (MLV), in addition to testing and removing PI animals, is used to reduce virus circulation in the herd (Moennig and Greiser-Wilke, 2003). In the U.S., reliance is placed on detection and removal of PI animals and vaccination of breeding animals before conception. Recent research suggests that, to be effective, BVDV vaccines should contain at least a BVDV 1 and a BVDV 2 strain (Fulton et al., 2003).

BVDV research as summarized above has benefited from the efforts of a cadre of outstanding scientists and diagnosticians, as reported and illustrated by the authors of the preceding chapters. However, BVDV has proven to be a difficult and challenging subject and despite these efforts much remains to be done. As this book goes to press there is gathering momentum for the development and implementation of a national program for BVDV control in the United States. Although eradication of BVDV in the U.S. may be a difficult goal to achieve, the reduction of the losses currently caused by BVDV is a readily attainable goal and is well worth pursuing. Ideally, the drive to reduce BVDV losses would be multifaceted and widely implemented. It would require the cooperation of producers, practitioners, diagnosticians, and regulators, and a refocusing of research and control efforts.

REFOCUSING BASIC RESEARCH

NONCYTOPATHIC BVDV

To date, heavy emphasis has been placed on examining differences between cytopathic and noncytopathic viruses *in vitro*. While intriguing from a scientific standpoint, such research does little towards reducing the incidence of BVDV in the field. Cytopathic viruses are rare and isolated only in association with mucosal disease or postvaccinal disease resulting from inoculation with a cytopathic BVDV vaccine. Mucosal disease, although an interesting phenomenon, is not a source of major economic loss for producers. The major economic impact of BVDV infection is the result of losses associated with reproductive or respiratory disease, which are almost always the result of infection with a noncytopathic virus. In contrast, no clinical outbreaks of acute disease have been traced to infection with a cytopathic BVDV.

Immunosuppression and acerbation of secondary infections, associated with infection with noncytopathic BVDV, also contribute to economic losses. There is no evidence to indicate that acute, uncomplicated infections with cytopathic BVDV are more clinically severe than infection with noncytopathic BVDV. In fact, the most clinically severe forms of acute BVDV infections are associated with noncytopathic BVDV. Thus, comparing cytopathic and noncytopathic viruses *in vitro* yields little information that would help limit BVDV infections *in vivo*. Efforts to limit the damage caused by BVDV infections would be better served by research that addresses the real significance of noncytopathic

BVDV strains and the host's response to them. In addition, the nature of immunosuppression and of protective immune response engendered by BVDV should be studied. Because noncytopathic virus may establish persistent infections, all available BVDV vaccines contain only cytopathic BVDV. It is not known, however, if cytopathic BVDV contained in vaccines are any safer than the noncytopathic viruses, especially for fetuses.

T-CELL RESPONSES

Considerable amount of information is available on B-cell immune response to BVDV vaccines, but little information is available regarding the T-cell responses. This is partly due to the availability of simple and reliable technology to examine and compare B-cell responses, as expressed by neutralizing antibodies present in serum. Unfortunately, such technology is not yet available for comparing T-cell responses, although it appears that the T-cell response is as important, if not more important, as B-cell response in the development of acquired immunity against BVDV. Research that focuses on methods to improve T-cell response to vaccination is sorely needed. This is dependent upon developing simple, robust, and reliable methods for measuring and comparing T-cell responses in infected, vaccinated, and nonvaccinated animals.

ACUTE, PROLONGED INFECTIONS

Historically, BVDV infections have been categorized as acute or persistent. Persistent infections are defined as lifelong infections resulting from *in utero* exposure of the animal to a noncytopathic BVDV. Lifelong infections result because the animal develops a specific immune tolerance for the virus to which it was exposed *in utero*. Acute infections, on the other hand, are defined as infections in which the immune system clears the virus within 14 days. However, the real picture is somewhat more complicated. For example, some animals exposed after birth (acute infection) may mount an immune response but fail to clear the virus within 14 days, some animals may clear the virus after a prolonged viral shed, and others may allow the virus to replicate in immunologically privileged sites such as testes and ovaries (see Chapter 9).

The damage resulting from acute infections may not be over after the virus has been cleared. Thus, there may be lingering problems that are not detected until several months after viremia has passed. Prolonged infections and persistent infections within privileged sites also contribute to the spread

of BVDV in cattle populations. Current surveillance programs, designed to detect classic persistently infected animals, will invariably miss these types of infections. In addition, the true cost of BVDV must factor in the problems in production that occur after the virus has been shed. This is difficult to track because onset of acute infection and its repercussions on animal production may be months apart.

LOW-VIRULENCE STRAINS OF BVDV

It is easy to determine the impact of infection with highly virulent strains of BVDV. The impact of low-virulence strains, on the other hand, is difficult to ascertain. However, infection with these strains should not be considered a benign event even though their effects may not be immediately apparent. Infection with low-virulence strains of animals that are actively laying down bone and muscle tissues may have long-term effects on their growth potential. In addition, low-virulence strains may lead to immunosuppression, setting up the animal for more severe secondary infections.

The impact of low-virulence strains on vulnerable populations of cattle should also be studied in detail. Examples of vulnerable animals include the fetus, weaned animals from which passive antibodies have disappeared or are waning, animals newly introduced into the herd, and stressed animals. Biosecurity to prevent the introduction of BVDV in animal herds and adequate vaccination programs are particularly important to limit infection in vulnerable animals. The significance of BVDV isolates from giraffe, antelope, and reindeer also need to be studied.

REFOCUSING CONTROL EFFORTS

Historically, BVDV control efforts have been based on vaccination. However, 40+ years of vaccination have not resulted in a significant decrease in BVDV losses nationwide. Although vaccination can be an effective component of a BVDV control plan, it is not a stand-alone answer to the problem. For a control effort to be effective, proactive management plans must be in place, and the development of such plans requires a complete understanding of BVDV transmission within populations, surveillance strategies, biosecurity on the farm, and the role of vaccination.

TRUE IMPACT OF BVDV

The compelling reason for a BVDV control program is that reducing BVDV infections is cost-effective. This premise assumes that the expenses incurred in

preventing BVDV infections are offset by savings realized by more efficient animal production. Although most producers agree that BVDV infections are detrimental, the full extent of BVDV's impact on production is frequently underestimated. In a layman's mind, BVDV-associated reproductive disease presents as the birth of PI animals. However, the birth of PI animals following the introduction of BVDV into a group of dams may represent just the tip of the iceberg. Clinical presentation of BVDV-associated reproductive disease also includes failure to conceive (repeat breeding), abortion, mummification, congenital abnormalities, and the birth of neonates that appear normal but fail to thrive. In order to determine the true impact of BVDV reproductive disease, animal producers and clinicians need to be alert to the many different forms of BVDV-induced reproductive problems.

BIOSECURITY ON THE FARM

Vaccination is a component of the control plan rather than an end unto itself and vaccines should not be considered "silver bullets" in the eradication of BVDV. In the absence of a good management program, which by definition would include a biosecurity plan, protection via vaccination will eventually fail. Preventing long- and short-term exposures to BVDV by limiting the introduction of BVDV into a herd is as important as protecting the animals by vaccination. Thus, PI animals must be removed and new additions to the herd should be isolated until their BVDV-free status has been established. In the case of bred heifers, BVDV-free status cannot be assured until the calf has been tested. Care should be taken to assure that a good BVDV management program is in place for all parties when facilities and equipment are shared between production units. A producer's biosecurity program is only as good as that of the neighbor he shares a fence with or the fellow exhibitor with whom he shares a show ring.

DISEASE SURVEILLANCE

The present testing for BVDV is sporadic and is usually done in response to an outbreak of the disease and focuses on the detection of PI animals. However, exposure to BVDV may result in subclinical disease that may impact profitability but is not immediately apparent from casual observation of the herd. Routine BVDV surveillance programs would be more effective at detecting and controlling BVDV outbreaks. Further, not all outbreaks of BVDV-related disease can be traced to contact with a PI animal. PI animals are certainly a major factor

in keeping BVDV in circulation, but acute BVDV infections cannot be ignored for surveillance programs to be effective. As discussed above, non-PI animals may experience prolonged viremia or may have localized infections that are not cleared. Further, aborted or stillborn fetuses may serve as sources of BVDV infection for a herd. Effective control programs must include the use of reliable tests for the detection of both acute and persistent infections and examination of aborted and stillborn fetuses.

VACCINE DESIGN

When developing vaccines, one of the questions that should be asked is whether the strains used in the vaccine reflect and protect against viruses the animal will be exposed to in the field. The first BVDV vaccines were produced in the 1960s. Viral strains used in these vaccines—e.g., Singer, NADC, NY-1, and C24V (Oregon strain), are still found in vaccines 40 years later. Retrospective analysis of these strains has shown that all of them belong to a single branch of the pestivirus family tree—e.g., BVDV genotype 1. In the early 1990s, a newly recognized group of BVDV field isolates, eventually termed *BVDV genotype 2*, was found to break through BVDV vaccination programs. A second generation of BVDV vaccines that contain the old vaccine strains plus strains from the BVDV 2 genotype are now becoming available. Time will tell whether these vaccines are more efficacious than the old ones.

As more BVDV strains are characterized, subgenotypes of BVDV are being recognized within both BVDV 1 and BVDV 2 genotypes. Thus, BVDV 1 has been subdivided into BVDV 1a and BVDV 1b. Recent surveys in the U.S. have shown that BVDV 1b is found most frequently in association with clinical disease and that most BVDV 1 strains isolated from the field belong to the BVDV 1b subgenotype. However, when BVDV 1 strains used in vaccines were characterized, it was found that they predominantly belonged to the BVDV 1a subgenotype. The practical significance of this finding is not clear and it is also not known whether the inclusion of BVDV 1b in vaccines would offer better protection. Further research is needed to answer these questions.

The observation of incomplete fetal protection following prebreeding vaccination should also be examined. Currently used vaccines are evaluated by determining whether they protect against BVDV-induced respiratory disease. Although some vaccine manufacturers have started to evaluate their vaccines for fetal protection, more needs to be done in this

area. Current vaccines with fetal protection label claims were licensed based on a one-time challenge of BVDV. Animals in contact with PI animals will be challenged by virus shed by the PI on a daily basis. It is not known how well vaccination stands up to such repeated challenges. Further, fetal protection appears to be dependent upon the prevention of viremia, not reduction of viremia or clinical disease. Thus the “perfect” BVDV vaccine would prevent infection rather than disease and induce both cellular and humoral immune responses.

EFFECTS OF STRESS ON VACCINE EFFICACY

Vaccines are commonly administered when animals are gathered for weaning, sorting, branding, and shipping. Unfortunately, the animal is under stress from a number of different factors at these times and its immune response is not functioning at its peak. Vaccination at these times may not result in an optimum immune response and may even cause transient losses in production. Thus, management practices may impact on the efficacy of the vaccine and should be taken into account when implementing a control program with vaccination.

OTHER QUESTIONS

The recognition of severe acute BVD in the early 1990s changed our perception of BVDV-induced disease severity. It is important to continue to monitor the role of newly emerging strains of BVDV on disease severity and on acute and persistent infections. The role of vaccination in the emergence of new BVDV strains should be monitored, and the current methods for the detection of BVDV in semen samples need improvement. The development of a prenatal test to determine the existence of PI animals would also be useful.

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