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Review

Approved and experimental countermeasures against pestiviral diseases: Bovine viral diarrhea, classical swine fever and border disease

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ABSTRACT

The pestiviruses, bovine viral diarrhea virus (BVDV), classical swine fever (CSFV) and border disease virus, are important livestock pathogens in many countries, but current vaccines do not completely prevent the spread of infection. Control of pestiviral diseases is especially difficult due to the constant viremia and viral shedding of persistently infected (PI) animals, which must be identified and eliminated to prevent disease transmission. Existing vaccines are limited by the delay between vaccination and the onset of protection, the difficulty of differentiating serologically between vaccinated and naturally infected animals and the need for broad vaccine cross-protection against diverse virus strains. Antiviral therapy could potentially supplement vaccination by providing immediate protection in the case of an outbreak. Numerous compounds with *in vitro* antiviral activity against BVDV have been identified through its role as a surrogate for hepatitis C virus. Fewer drugs active against CSFV have been identified, but many compounds that are effective against BVDV will likely inhibit CSFV, given their similar genomic sequences. While *in vitro* research has been promising, the paucity of efficacy studies in animals has hindered the commercial development of effective antiviral drugs against the pestiviruses. In this article, we summarize the clinical syndromes and routes of transmission of BVD, CSF and border disease, discuss currently approved vaccines, review efforts to develop antiviral therapies for use in outbreak control and suggest promising directions for future research.

Contents

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1. Introduction

Bovine viral disease virus (BVDV), classical swine fever virus (CSFV) and border disease virus (BDV) are members of the genus *Pestivirus*, family *Flaviviridae*, that cause disease in wild and domestic animals worldwide (Figs. 1 and 2, Table 1). Although rapid diagnostic tests are available, the nonspecific and varied nature of these diseases has made outbreak control difficult. An epidemiological hallmark of the pestiviruses is their ability to infect the immuno-naïve fetus resulting in persistent infection. Persistently infected (PI) animals consistently shed infectious virus throughout their lives, which poses unique challenges to disease control. Though acute disease is not uncommon, infection by the ruminant pestiviruses often results in mild clinical signs, allowing the disease to spread undetected through the infection of the susceptible fetus and the creation of additional PI animals. Disease due to CSFV is generally more severe than that caused by BVDV or BDV, resulting in high morbidity and mortality and thus is subject to intense regulatory attention throughout much of the world. Minimizing transmission from PI animals through their identification and elimination is the central tenet to limiting pestiviral disease.

Vaccines for BVDV and CSFV have been used commercially for the better part of a century but are not sufficient by themselves to control pestiviral diseases. Effective vaccines must consistently protect against congenital infection to limit transmission of the viruses *in utero*. For BVDV, the presence of multiple genotypes and subtypes dictates the need for broad crossreactivity to protect against challenge by multiple viral strains. For CSFV, the ability to differentiate between vaccinated and naturally infected animals (DIVA) is crucial for regulatory concerns and disease surveillance and is the biggest hurdle to the implementation of widespread vaccination in the case of disease outbreaks in areas currently free of the disease. These issues have been the focus of intense study and vaccines currently available or in development should enhance the efficacy of pestiviral disease control.

Antiviral compounds represent a potential stop-gap measure where vaccination has not been practiced or has proven ineffective. The attractiveness of antiviral drugs in the control of pestiviral disease lies largely in their potential to provide immediate protection to at-risk animals in the case of a disease epidemic. However, although several antiviral drugs are in development, none is currently licensed for use in livestock. We recently reported the use of a novel antiviral compound to markedly reduce levels of viremia in calves PI with BVDV, which we propose as the most stringent *in vivo* test for an antiviral compound (Newcomer et al., 2012a). As described below, additional compounds are being explored for use in CSFV outbreak control. The efficacious use of antiviral drugs has the potential to limit economic losses, animal death and suffering and trade disruptions associated with pestiviral disease. Continued development of promising compounds is needed to achieve availability for field use.

The goal of this paper is to evaluate the current and future use of vaccines and antiviral drugs as countermeasures to BVDV, CSFV and BDV. The clinical features and transmission pathways of the respective disease syndromes are reviewed as they relate to disease control. We discuss the current use of vaccines for each disease and review the current status of available vaccines as well as novel vaccine strategies under development. The use of antivirals to limit pestiviral disease is explored and the results of *in vitro* and *in vivo* testing are reviewed.

2. Bovine viral diarrhea

2.1. Clinical signs and transmission

2.1.1. Transient infection

Clinical disease caused by BVDV infection can take several forms and is a source of significant economic losses in cattle worldwide (reviewed by Walz et al. (2010)). The clinical effects of BVDV may manifest in any of several body systems of affected cattle although reproductive losses associated with infection are thought to have the largest negative economic impact for producers (Grooms, 2004). Viral strains are classified into either cytopathic (CP) or noncytopathic (NCP) biotypes based on the presence or absence, respectively, of vacuolation and cell death in cultured epithelial cell lines following infection. Biotypes are not an accurate predictor of pathogenicity as both have been responsible for severe disease outbreaks.

Acute infection occurs when seronegative, immunocompetent cattle are exposed to the virus. Primary viremia may last up to

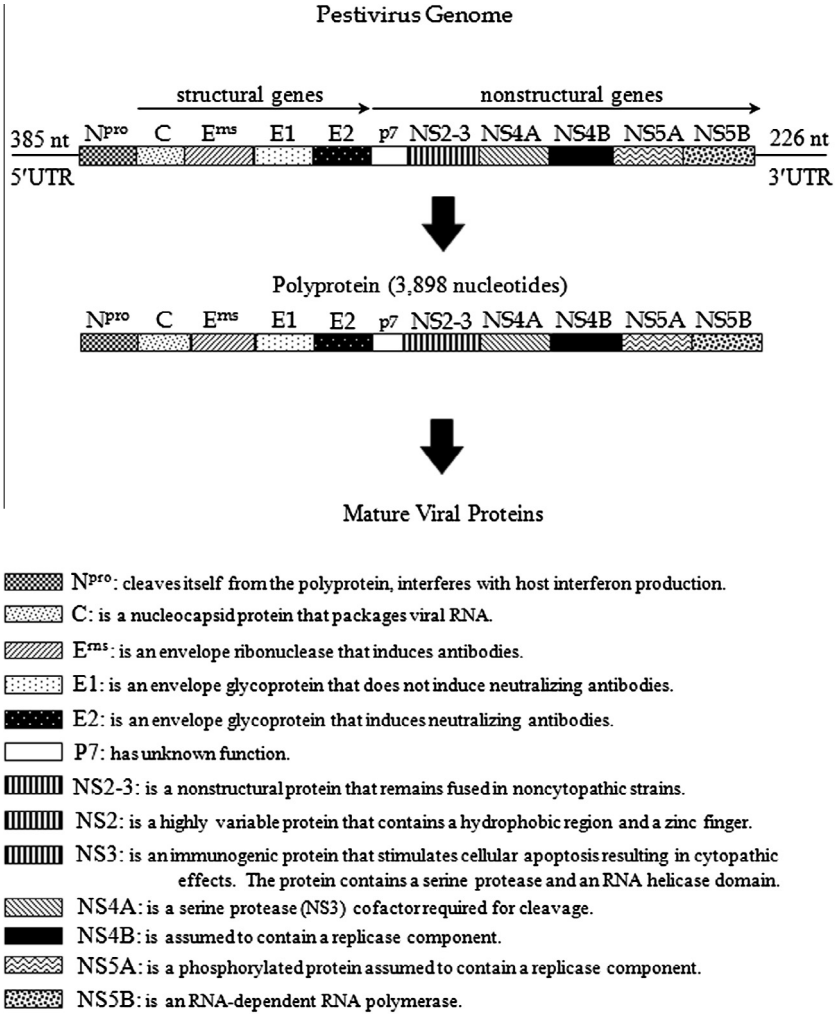

Fig. 1. Genomic organization of the pestiviruses. The genome encodes a single polypeptide that is subject to co- and post-translational processing that yields up to 12 mature proteins. nt = nucleotides; UTR = untranslated region.

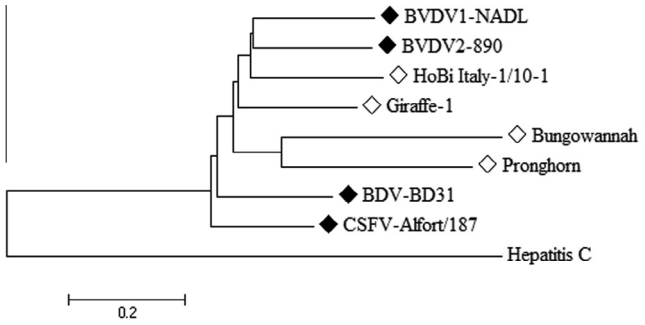

Fig. 2. Relatedness of Pestivirus and Hepacivirus species based on sequence homology of the region encoding the 5′ UTR to E2. The evolutionary history was inferred using the Neighbor-Joining method. Branch lengths are proportional to genetic distances. Pestivirus member species are marked with a solid diamond (◆) while proposed pestivirus species are marked with an empty diamond (◇). Figure provided by Fernando Bauermann, Department of Preventive Veterinary Medicine, Virus Section, Federal University of Santa Maria, Santa Maria, Rio Grande do Sul, Brazil.

15 days and is often accompanied by pyrexia and leukopenia (Stoffregen et al., 2000; Liebler-Tenorio et al., 2002). Clinical signs due to acute BVDV infection include depression, inappetance, decreased milk production, oculonasal discharge and oral ulcerations. Despite its name, acute BVDV infection resulting in diarrhea is poorly characterized and inconsistently seen, particularly in adult cattle. The contribution of BVDV infection to respiratory disease is not well understood but is thought to be due to its immunosuppressive qualities that enhance the pathogenesis of other viral or bacterial pathogens. Consequently, BVDV has been implicated along with other pathogens (e.g., *Pasteurella multocida, Manheimia hemolytica*, parainfluenza-3 virus, bovine respiratory syncytial virus) in the pathogenesis of the bovine respiratory disease complex (Fulton et al., 2000; Ridpath, 2010a). Acute BVDV infection may also result in severe thrombocytopenia and hemorrhage with high resultant mortality (Pellerin et al., 1994). Based on genomic and antigenic differences, isolates from the original outbreaks of severe hemorrhagic BVDV infection were determined to belong to a separate genotype, BVDV2, than previously recognized strains.

As with other pestiviruses, BVDV is able to readily cross the placenta of pregnant animals and infect the fetus (Fray et al., 2000). Pathogenesis is largely determined by the age of the fetus when infected. A naïve cow infected during the first month and a half of gestation may suffer early embryonic death, possibly due to endometrial inflammation resulting from the viral infection. Later in gestation, BVDV infection can result in central nervous system malformations due to the ability of BVDV to cross the blood–brain barrier. Cerebellar hypoplasia is the most notable abnormality seen, while other congenital defects include hydrancephaly,

Table 1
Classification, virion structure, replication cycle and genetic diversity of the pestiviruses.

Classification: The *Pestivirus* genus is one of three genera that together with the flaviviruses and hepaciviruses comprise the family *Flaviviridae*. Member viruses are animal pathogens responsible for a variety of clinical syndromes. The pestiviruses differ from other *Flaviviridae* by the presence of the N^{pro} autoprotease, which is the first protein encoded in the open reading frame (ORF).
Virion structure: The pestivirus genome consists of a single strand of positive-sense RNA approximately 12.3 kb in length that encodes a single ORF of about 4,000 codons, and is neither capped nor polyadenylated Becher et al. (1998). The lipid envelope is formed during maturation and assembly of the virion by budding through membranes of infected cells.
Virus replication: Cellular attachment is thought to be mediated primarily through the interaction of a pestiviral envelope protein with glycosaminoglycans in the host-cell membrane Iqbal et al. (2000). After attachment, binding of a second viral envelope protein triggers receptor-mediated endocytosis, that delivers the genome into the cytosol through a pH-dependent step. After entry to the cytosol, the approximately 380-nucleotide 5′ UTR serves as the internal ribosomal entry site. A single translated polyprotein is subject to co- and post-translational processing that yields 11–12 mature proteins, four of which are structural Collett et al. (1988) (Fig. 1). Complementary RNA strands are present four to six hours following infection, with the accumulation of positive-sense strands exceeding negative-sense strands Gong et al. (1996). Pestivirus maturation is believed to occur within intracellular vesicles, which are subsequently released via exocytosis. Maximum release of infectious virus generally occurs 12–24 h after infection.
Genetic diversity: Bovine viral diarrhea virus (BVDV), which exists as two genotypes, serves as the exemplar of the genus, which also includes classical swine fever (CSFV) and border disease virus (BDV). Newer proposed members of the genus include pronghorn virus, Bungowannah virus and the Hobi-like viruses. Sequence homology is very high between the BVDV genotypes and between CSFV and BDV, while the newer agents are generally more divergent (Fig. 2).

micropthalmia, and ocular cataracts (Blanchard et al., 2010; Otter et al., 2009). Fetal infection at the appropriate stage of gestation may result in persistent infection (see below).

In 1998, a new face was added to the multifaceted spectrum of clinical BVDV syndromes when a seropositive, nonviremic bull at an artificial insemination (AI) center was found to have a unique, localized persistent testicular infection (PTI) (Voges et al., 1998). Since then, only one other report exists of a similarly infected bull, also found at a bull stud (Givens et al., 2012b). Experimental studies following the description of the original bull with PTI have been unable to reproduce the exact syndrome although similar results have been achieved (Givens et al., 2003b). The prevalence of bulls with PTI is thought to be very low.

2.1.2. Persistent infection

Of utmost importance in the propagation of BVDV infection is the PI animal. In utero exposure to NCP strains before development of fetal immunocompetence (generally by 125 days of gestation) can result in a PI calf. Affected calves are often weak at birth and the majority will die before one year of age. However, others may not show signs of disease but continuously shed virus and are important in the epidemiologic aspects of BVDV propagation (Brock, 2003). Approximately 0.3% of the animals entering the feedlot are thought to be PI animals (Loneragan et al., 2005). Superinfection of PI calves with homologous CP strains of BVDV may result in mucosal disease (MD) (Bolin, 1995). The prevalence of MD is very low but is accompanied by very high mortality. Most calves with MD have widespread ulceration of the upper gastrointestinal tract as well as hemorrhagic lesions in the abomasum and elsewhere.

2.1.3. Transmission

The PI animal is the primary reservoir of BVDV and serves as the major source of infection to other cattle through direct contact with infected material. Infectious virus is secreted in nasal discharge, saliva, tears, urine, feces, milk and semen. Transmission can also occur through breaks in the skin and by mechanical means including personnel, equipment and arthropod vectors (Niskanen and Lindberg, 2003). Airborne transmission without direct contact may occur over very short distances but only for a limited time period. Artificial insemination is a potential route of transmission, as infectious semen may remain contaminated even after cryopreservation and processing (Gard et al., 2007). Several feral ruminant species are susceptible to BVDV infection and transmission to cattle has been documented in specific experimental situations (Negron et al., 2012). However, interspecies transmission is largely uncharacterized and sylvatic reservoirs remain to be proven a significant threat to domestic livestock.

2.2. Diagnosis

Due to the diverse clinical and subclinical syndromes displayed by infected animals, a definitive diagnosis of BVDV infection is only made through laboratory testing. A number of assays are available, and selection of the appropriate test will be dictated by several factors, including the management system of the affected farm, financial constraints, and availability of validated tests at a given laboratory (Edmondson et al., 2007). Since not all available tests are appropriate for each clinical situation, care should be taken when selecting an assay, in order to reach a valid diagnosis quickly and efficiently (Saliki and Dubovi, 2004). The identification and isolation of PI animals is the most critical step in the control of BVDV; most testing therefore focuses on the detection and identification of PI animals, to remove them from the herd and to limit virus shedding and spread. However, not all tests are appropriate for identification of PI animals.

Essentially all diagnostic tests for BVDV fall into one of four main categories: antigen detection, molecular techniques, virus isolation and serology. Direct antigen detection assays are relatively quick and economical to perform and represent the most widely used tests for the identification of PI animals (Saliki and Dubovi, 2004). Immunohistochemistry (IHC) performed on fresh or formalin-fixed tissue samples, commonly taken from the ear, represent a common assay used to detect BVDV antigen although alternate sample sites may be equally as sensitive (VanderLey et al., 2011). More recently, commercial antigen-capture ELISA kits have been developed that rely on monoclonal antibodies targeting the E^{rns} glycoprotein of BVDV. Not every strain of BVDV can be detected by these tests (Gripshover et al., 2007). Pragmatically, we routinely recommend the collection of ear notch samples in herds dealing with clinical signs of BVDV infection and in herds with a history of the disease as a means of continued monitoring for the presence of PI animals.

Molecular detection techniques have also become a valuable tool to detect PI animals in suspected cases of BVDV. Commercially available kits with simple viral RNA extraction steps have encouraged the acceptance of quantitative reverse-transcription PCR (qRT-PCR) as the primary herd screening assay used by many diagnostic laboratories for the detection of PI animals. The sensitivity and specificity of the qRT-PCR assay are high and a variety of samples can be used, including serum, whole blood, milk, tissues, nasal swabs, semen and embryos. Occasionally, positive qRT-PCR results are due to acute infections; thus, valuable animals should be retested in 30 days to confirm the animal's PI status. Where available, qRT-PCR can be used in much the same manner as antigen detection techniques for the identification of PI animals with the added benefit of being able to pool samples for more economical

testing (Weinstock et al., 2001). However, the pooling of samples lowers the sensitivity of the assay and thus can be controversial if the protocols have not been rigorously validated. A non-invasive testing method for the detection of PI animals by submitting consumption surface swabs for qRT-PCR analysis has been developed in our laboratory, allowing the screening of large groups with minimal expenditure of labor (Givens et al., 2011). Given the high sensitivity of molecular techniques, we expect the assays will play a continuously larger role in the control of BVDV through the identification of PI animals.

Isolating virus from tissues or secretions of infected animals has historically been the "gold standard" diagnostic test for BVDV. However, the procedure is time consuming, relatively expensive and a single test is incapable of differentiating persistent and acute infections. Since identification of PI animals is critical for the control of BVDV, virus isolation techniques have largely been supplanted by newer assays more suitable to the detection of PI animals. The value of virus isolation lies in the detection of acute infection in the individual animal showing clinical signs of disease or to confirm the virus as the cause of an acute disease outbreak. Isolation of virus from a clinically affected animal represents active infection and the source of infection (e.g., PI animals) should then be identified.

Serologic testing as a diagnostic tool for BVDV is of limited value where vaccination is practiced, due to the difficulty of differentiating between infected and vaccinated animals (DIVA). However, serologic testing is invaluable in eradication efforts where vaccination is no longer practiced. It may also be beneficial to assess vaccine efficacy or compliance with a vaccine protocol or to assess the herd's exposure status. Persistently infected animals do not mount an antibody response to the infecting strain of the virus; therefore, serologic tests do not identify PI animals. Consequently, in BVDV endemic areas, we recommend the use of IHC, antigen-capture ELISA or qRT-PCR for routine herd surveillance.

2.3. Vaccines

2.3.1. Role of vaccines in BVDV control

The essentials of BVDV control are the minimization of transmission of infectious virus from infected to susceptible animals and the elimination of viral reservoirs. Vaccination has been practiced for decades and yet the disease remains a serious global concern due to incomplete protection or lack of efficacious use of available vaccines. Vaccination programs for BVDV should aim to prevent viremia, acute disease and reproductive losses in vaccinated animals and prevent the creation of PI animals. From the point of view of BVD control, preventing PI animals is the most crucial goal, as these animals serve as the reservoir of the virus and are the main source of new infections in susceptible cattle.

Challenges to BVD control programs based on vaccination include the delay of onset of protection following vaccination, the need to protect against a wide array of viral genotypes and subgenotypes, and the requisite for fetal protection. Therefore, vaccination is simply one element of pestiviral control and should be used in conjunction with the identification and removal of PI animals, effective biosecurity management and detection of new infections (Stahl and Alenius, 2012; Ridpath, 2012). Detection of new infections by the use of serologic tests is complicated by vaccination as conventional vaccines do not allow DIVA. However, the detection of transiently infected animals is less crucial to the control of BVDV than the detection of PI animals. Consequently, there has not been extensive pressure to develop DIVA-adherent BVD vaccines although the development of such vaccines would greatly assist eradication efforts by enabling serologic detection of infected animals while continuing to provide vaccinal protection against exposure. Vaccination is an important aid in the control of BVD, as evidenced by the more than 175 vaccines licensed in the United States in either modified-live or inactivated formulations (Kelling, 2004).

2.3.2. Inactivated vaccines

Inactivated BVDV vaccines have long been used in veterinary medicine as either stand-alone preparations or in combination with other viral and/or bacterial antigens. Safety is the major advantage of inactivated vaccines, which can generally be assumed to be safe for use in pregnant cattle with no or unknown vaccination history. Because the viral antigen is incapable of replication, infection of the fetus does not occur, lowering the risk of abortion and the creation of PI animals. A major drawback of inactivated vaccines is the need for initial vaccinates to be dosed twice at 2- to 4-week intervals; lack of compliance among producers can leave cattle susceptible to subsequent viral challenge. Peak immunity from inactivated vaccines is seen only after the initial dosing schedule has been completed. Therefore, a delay of 4–6 weeks from the time of initial vaccination may occur before optimal protection is realized. In the case of an outbreak in naïve animals, this delay may prove costly and the use of live, attenuated vaccines may be a better alternative.

2.3.3. Live, attenuated vaccines

In general, modified-live (MLV) vaccines stimulate higher production of neutralizing antibodies and longer duration of protection than inactivated vaccines (Cortese et al., 1998b; Ridpath et al., 2003). In addition to inducing antibody production, MLV vaccination also stimulates cell-mediated immunity (Platt et al., 2009; Woolums et al., 2013). A rapid onset of protection is seen after MLV vaccination as evidenced by partial protection from experimental BVDV challenge seen three days after vaccination and complete protection seen after 5–7 days (Brock et al., 2007). Consequently, the use of MLV vaccines can be expected to provide rapid protection after only a single dose. While inactivated vaccines have historically been used in pregnant cattle, within the past 10 years, several MLV vaccines have been developed that are labeled for administration to pregnant cattle on the condition that they have been vaccinated with the same vaccine within the previous 12 months according to the label instructions. It is our preference to use MLV vaccines when such conditions are met or when vaccinating non-pregnant cattle. However, in situations where this is not feasible, notable protection against BVDV challenge can be achieved with two doses of an inactivated vaccine.

2.3.4. Multivalent, cross-protective vaccines

Isolates of BVDV can be divided into two different genotypes and numerous subtypes, based on genetic and antigenic differences (Fig. 3). Consequently, the ability of BVDV vaccines to cross-protect against multiple common genotypes and subgenotypes is essential (Kelling, 2004; Chase et al., 2004). Phylogenetic analysis reveals significant differences in sequence homology between BVDV1 and BVDV2 and both genotypes can be subdivided into subgenotypes (Vilcek et al., 2001; Ridpath, 2005). Historically, BVDV vaccines have been comprised of BVDV1a isolates; consequently, vaccinal protection against BVDV2 has been studied following the administration of monovalent BVDV vaccines containing type 1 isolates. Several studies have demonstrated production of virus-neutralizing antibodies to BVDV2 following vaccination with monovalent MLV BVDV1 vaccines (Cortese et al., 1998b; Bolin and Ridpath, 1989) or inactivated preparations (Hamers et al., 2002; Fulton and Burge, 2000). Protection from experimental BVDV2 challenge following vaccination with BVDV1 vaccines has also been demonstrated (Dean and Leyh, 1999; Makoschey et al., 2001; Hamers et al., 2003; Fairbanks et al., 2003; Kelling et al., 2007).

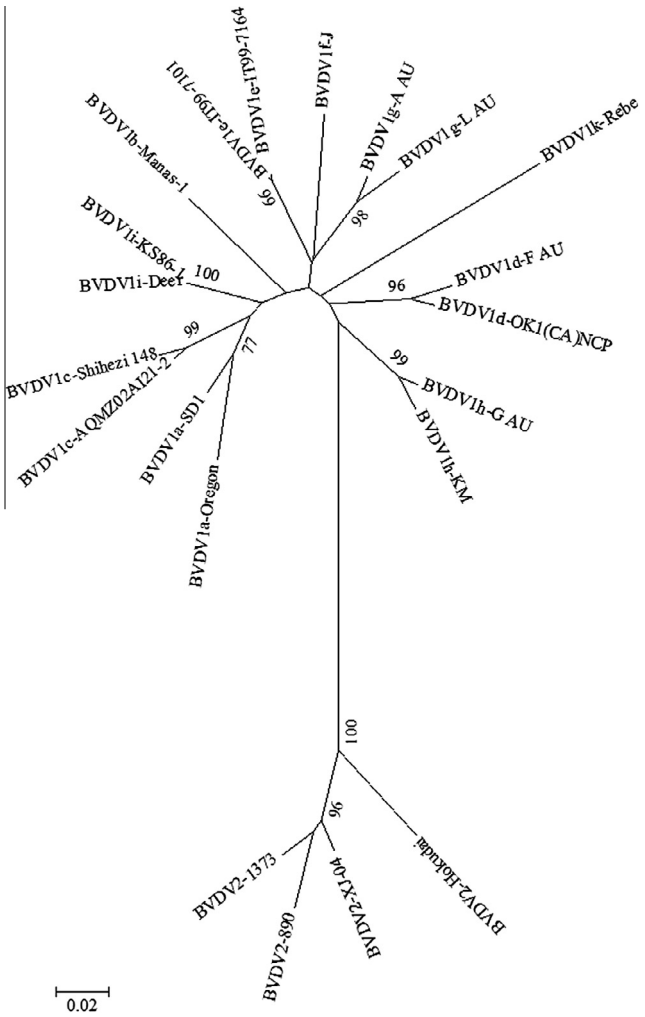

Fig. 3. Diversity of selected strains of bovine viral diarrhea virus based on genetic homology of the 5′ UTR. The evolutionary history was inferred using the Neighbor-Joining method. Branch lengths are proportional to genetic distances. Figure provided by Fernando Bauermann, Department of Preventive Veterinary Medicine, Virus Section, Federal University of Santa Maria, Santa Maria, Rio Grande do Sul, Brazil.

While BVDV1a isolates are most often used as vaccine strains, the predominate field strains in North America are of subtype 1b; cross-protection between the subgenotypes has been demonstrated following experimental challenge (Fulton et al., 2003b; Xue et al., 2010; Palomares et al., 2012). However, other studies have demonstrated that protection against heterologous strains is inferior to homologous strains or inadequate to prevent infection (van Oirschot et al., 1999; Van Campen et al., 2000; Fulton et al., 2003a). Because of this increased concern, many vaccines now contain BVDV2 isolates in addition to the BVDV1 strains which have historically been the mainstay of BVDV vaccines (Beer et al., 2000; Kovacs et al., 2003; Fairbanks et al., 2004). Inclusion of the two viral genotypes in a MLV vaccine generally provides superior protection against the varied BVDV isolates to which the individual may be exposed. We recommend that such vaccines be used to help prevent infection by varied strains encountered in the field.

Recent research has focused on the ability of vaccination to prevent fetal BVDV infection, following reports of transplacental transmission in vaccinated animals (Van Campen and Woodard, 1997; Van Campen et al., 2000). As PI animals represent the main reservoir of BVDV, eliminating such infections is key to controlling the disease and its transmission. Experimental challenge after vaccination has demonstrated substantial, albeit incomplete fetal protection. Using monovalent MLV vaccines, fetal protection rates have ranged from 83% to 92% when challenged with homologous virus (Cortese et al., 1998a; Dean et al., 2003). However, in a similar study in which vaccinated animals were challenged with heterologous virus, fetal protection was seen in only approximately 60% of vaccinates (Brock and Cortese, 2001). Fetal protection is also variable following vaccination with an inactivated vaccine, ranging from 36% to 100% (Brownlie et al., 1995; Zimmer et al., 2002; Grooms et al., 2007; Rodning et al., 2010). Recent studies examining the efficacy of multivalent MLV vaccines have proved promising, with fetal protection consistently exceeding 85% (Ficken et al., 2006; Rodning et al., 2010; Xue et al., 2011; Leyh et al., 2011; Givens et al., 2012a). BVDV vaccines should therefore contain multiple strains to provide optimal protection from fetal infection.

2.4. Antiviral drugs

2.4.1. Drug targets in the pestivirus replication circle

The pestiviruses in general, and BVDV in particular, present several potential targets for directed antiviral therapy. The RNA-dependent RNA polymerase (RdRP), encoded by NS5b, is well characterized and the most frequent target of antiviral compounds (Choi et al., 2004). Several compounds with demonstrated *in vitro* activity against BVDV have been shown to exert their effect through antagonism of the RdRP (Manfredini et al., 2004; Angusti et al., 2008; Tonelli et al., 2010a; Newcomer et al., 2013b). The NS3 protein encodes a helicase and a serine protease and is a necessary component of the replication complex required for viral RNA replication, thus representing another target for antiviral therapy (Chaudhuri et al., 2012; Lawitz et al., 2013). Another potential, albeit nonspecific, target of antiviral drugs are the endoplasmic reticulum (ER) glucosidases, which are essential for virion processing and packaging (Alonzi et al., 2009). Inhibition of inosine-monophosphate dehydrogenase (IMPDH) has also been targeted by *in vitro* studies (Stuyver et al., 2002; Buckwold et al., 2003; Yanagida et al., 2004). In addition to known targets, the mechanism of action of several compounds with demonstrated antiviral efficacy against BVDV remains to be elucidated, potentially leading to the identification of additional targets.

The lack of proofreading capability of the RdRP holds practical consequences for the development of antiviral therapies. The high frequency of mutation allows for rapid selection of viruses with decreased sensitivity to antiviral compounds. Consequently, effective antiviral therapy is likely to require a combination of drugs. For hepatitis C (HCV), a closely related flavivirus that can also cause persistent infection, combination therapy with interferons and ribavirin has been shown to be more effective than monotherapy (Seeff and Hoofnagle, 2002) and formed the standard of care for treatment of most HCV infections until the approval of two HCV protease inhibitors by the FDA in 2011. Addition of either of the protease inhibitors to the ribavirin and pegylated interferon regimen results in higher sustained virologic response rates than to either ribavirin or pegylated interferons alone (Poordad et al., 2011; Jacobson et al., 2011). To date, reports of combination therapy for pestiviral infections are lacking. Synergistic effects are seen with combination therapy *in vitro* when iminosugars are combined with interferons alone (Ouzounov et al., 2002) or interferons and ribavirin (Woodhouse et al., 2008). Combination therapy involving nucleosidic compounds is effective at clearing cell lines infected with BVDV (Durantel et al., 2004; Woodhouse et al., 2008; Dukhan et al., 2005). Combination therapy will likely need to include drugs with complementary mechanisms of action to decrease the likelihood of selecting resistant viral mutants.

Table 2
Nucleosidic compounds with *in vitro* antiviral activity against bovine viral diarrhea virus (BVDV). Nucleosides are the most widely studied class of antivirals effective against the pestiviruses; they act through a variety of mechanisms. Abbreviations: EC(90) – effective concentration (90%); IFN – interferon; IMPDH – inosine-5′-monophosphate dehydrogenase; RdRP – RNA dependant RNA polymerase.

Compound	Findings	References
3′-Deoxyuridine	Demonstration of efficacy	Hollecker et al. (2004)
6-Methylmercaptopurine riboside	Antiviral activity antagonized by adenosine	Hoover and Striker (2008)
Adenine derivatives	Potential interaction with surface allosteric binding pocket on RdRP	Manfredini et al. (2004)
Azathioprine	More potent than mycophenolic acid	Stangl et al. (2004)
BDT substituted nucleosides	Substitution increases efficacy	Seio et al., (2004)
Beta-D-N(4)-hydroxycytidine	EC(90) = 2 μM	Stuyver et al. (2003)
Guanine nucleoside derivatives	Demonstration of efficacy	Dukhan et al. (2005)
Mizorbine	Synergism with IFN	Yanagida et al. (2004)
Methyl substuituted nucleosides	Demonstration of efficacy	Pierra et al. (2006)
Mycophenolic acid	Inhibition of IMPDH enzyme	Stuyver et al. (2002)
Pyrimidine nucleoside	Demonstration of efficacy	Ivanov et al. (2008)
Ribavirin	EC90 = 4 μM	Stuyver et al. (2003)
Ribavirin	Direct inhibition of viral replication	Escuret et al. (2002)
Ribavirin	Synergism with IFN	Buckwold et al. (2003)
Ribavirin	Synergism with IFN, iminosugars	Durantel et al. (2004)
Substituted adenine analogs	Inhibits BVDV RdRP in enzyme assays	Angusti et al. (2008)

2.4.2. Results of in vitro testing

As the *de facto* surrogate model for the evaluation of antiviral therapeutics effective against hepatitis C virus (HCV), BVDV has been widely studied as a test virus for novel antiviral therapies. Despite the invention of a self-replicating subgenomic HCV RNA replicon (Lohmann et al., 1999), surrogate testing using BVDV has continued due to the inability of the replicon assay to produce infectious virions and therefore identify compounds effective during the early (e.g., attachment and cell entry) or late (e.g., cell egress) steps of the viral replication cycle. Consequently, numerous compounds have been identified that possess specific or nonspecific *in vitro* antiviral activity against BVDV, but *in vivo* studies are lacking for most identified agents (reviewed by Finkielsztein et al. (2010)). Several nucleosidic compounds exhibit antiviral activity against BVDV *in vitro* (Table 2). The defined mechanisms of action of nucleosidic compounds, the most studied class of BVDV antivirals, are diverse and include chain termination (Hollecker et al., 2004), inhibition of the IMPDH enzyme (Yanagida et al., 2004) and inhibition of the RdRP (Angusti et al., 2008). Others may exert their effect as antimetabolites or through interaction with virus or host-cell proteins (Stuyver et al., 2003). The benefits of using compounds with different mechanisms of action are discussed above but as of yet, no data exist regarding the *in vivo* efficacy of nucleosidic compounds.

The iminosugars and the nitrogen heterocyclic drugs represent the two largest subgroups of the non-nucleosidic compounds. The mechanism of action of the iminosugars is through viral glycoprotein misfolding, mediated by inhibition of ER glucosidases (Alonzi et al., 2009). Several iminosugars have demonstrated *in vitro* activity against BVDV (Table 3) but have not yet been subjected to *in vivo* testing. Likewise, the antiviral effects of the nitrogen heterocyclic compounds have only been demonstrated *in vitro* (Table 4). Most such compounds for which the mechanism of action has been identified appear to exert their effect through inhibition of the RdRP. The application of these compounds and others (Table 5) to the control of BVD has been hindered by the paucity of *in vivo* data.

Antisense oligonucleotide therapy is a relatively new antiviral strategy. Viral replication is inhibited by interference with the translational machinery through the use of small interfering RNA

Table 3
Iminosugar compounds with *in vitro* antiviral activity against bovine viral diarrhea virus (BVDV). Iminosugars usually act as glucosidase inhibitors, leading to viral protein misfolding. Abbreviations: IFN – interferon.

Compound	Findings	References
Alkyl substituted deoxynojirimycin derivatives	Substitutions improve potency	Mehta et al. (2002)
Celgosivir	Enhanced efficacy in combination therapy	Whitby et al. (2004)
Deoxynojirimycin derivatives	Synergism with IFN	Ouzounov et al. (2002)
Deoxynojirimycin derivatives	Prevent formation and secretion of virus	Zitzmann et al. (1999)
Deoxynojirimycin derivatives	Clearance from cells in combination with IFN, ribavirin	Woodhouse et al. (2008)
Deoxynojirimycin derivatives	Glucosidase inhibition	Borges de Melo et al. (2006)
Deoxynojirimycin derivatives	Results in misfolding of envelope proteins and lack of E1-E2 complexes	Durantel et al. (2001)
Iminocyclitol compounds	Active against multiple enveloped viruses	Gu et al. (2007)

Table 4
Nitrogen heterocyclic compounds with *in vitro* antiviral activity against bovine viral diarrhea virus (BVDV). The nitrogen heterocycles are a diverse group of compounds that most commonly exert their antiviral effect by inhibiting or interacting with the viral RNA-dependent RNA polymerase (RdRP). Abbreviation: EC(50) – effective concentration (50%);

Compound	Findings	References
Acridine derivatives	EC(50) = 0.1–8 μM	Tabarrini et al. (2006), Tonelli et al. (2011)
AG 110	Inhibition of replication complexes	Paeshuyse et al. (2007)
Benzimidazole derivatives	Broad range of antiviral activity	Tonelli et al. (2008b), (2010b), Vitale et al. (2010), (2012)
Carboline derivatives	EC(50) = 0.26 μM	Sako et al. (2008)
Imidazopyridine derivatives	Interacts with BVDV RdRP	Chezal et al. (2010)
LZ37	Interacts with BVDV RdRP	Paeshuyse et al. (2009)
Quinoline derivatives	Inhibits BVDV RdRP	Carta et al. (2011)
Substituted pyrimidine derivatives	Inhibits BVDV RdRP	Paeshuyse et al. (2006), Puerstinger et al. (2006), (2007)
Thiazepine derivatives	Modest activity against BVDV	Struga et al. (2009)
VP32947	Inhibition of NS5B protein	Baginski et al. (2000)

Table 5
Miscellaneous antiviral compounds with *in vitro* activity against bovine viral diarrhea virus (BVDV). Abbreviations: EC(50) – effective concentration (50%); IFN – interferon; RdRP – RNA-dependent RNA polymerase.

Compound	Category	Findings	References
DB771, DB772	Aromatic cationic molecules	EC(50) = 0.15–0.8 μM	Givens et al. (2003a)
DB606, DB772, DB824	Aromatic cationic molecules	Elimination from fetal fibroblast cells	Givens et al. (2004)
DB772	Aromatic cationic molecules	Pan-pestivirus inhibitor	Newcomer et al. (2012a)
Geneticin	Aminoglycoside	Apparently interferes with viral assembly or release	Birk et al. (2008)
Aminoarylazo compounds	Azocompounds	EC(50) = 1.6–12 μM	Tonelli et al. (2009)
Arylazoenamines	Azocompounds	EC(50) = 0.8–10 μM,Interact with BVDV RdRP	Tonelli et al., 2008a, Giliberti et al. (2010)
SK3M4M5 M	Carboline derivatives	Likely target RdRP	Salim (2010) 520 /id)
Compound 1453	Cyclic urea derivative	Inhibits RdRP	Sun et al. (2003)
Cyclooxygenase inhibitors	Cyclooxygenase inhibitors	Additive to synergistic with IFN	Okamoto et al. (2009)
IFN alpha and gamma	Interferon	Suppression of replication	Bielefeldt-Ohmann and Babiuk (1988)
IFN alpha and gamma	Interferon	Growth suppresion by IFN alpha	Sentsui et al. (1998)
Human IFNs	Interferon	Demonstration of efficacy	Peek et al. (2004a), Buckwold et al. (2007)
BIT225	Ion channel inhibitor	Synergism with IFN and nucleoside analogues	Luscombe et al. (2010)
Artemisinin	Natural product	Additive with IFN and ribavirin	Romero et al. (2006)
Cantharidin, cephalotaxine and homoharingtonine	Natural product	Toxicity seen before evidence of efficacy	Romero et al. (2007)
Coronus didymus, Juglans australis, Lippia alba	Natural product	Demonstration of efficacy	Ruffa et al. (2004)
Petiveria alliacea	Natural product	Demonstration of efficacy	Ruffa et al. (2002)
Phyllanthus amarus root	Natural product	EC(50) = 33 μg/ml	Bhattacharyya et al. (2003)
Diphenylmethane analogs	Organic compound	EC(50) = 6.2–10.8 μ10 M	Hosoda et al. (2009), Salim (2010)
Hops derivatives	Oxygenated heterocycle	Efficacy likely due to presence of xanthohumol	Buckwold et al. (2004)
Coumarin derivatives	Oxygenated heterocycle	Suspected inhibition of RdRP	Mazzei et al. (2008), Giampieri et al. (2009)
Xanthohumol	Oxygenated heterocycle	Dose-dependent inhibition	Zhang et al. (2009)
Xanthohumol	Oxygenated heterocycle	Additive with IFN	Zhang et al. (2010)
Boronic acid analog	Peptidomimetic	Inhibitor of NS3 serine protease	Bukhtiyarova et al. (2001)
DPC-A69280–29	Thiazole urea class compound	Interferes with initiaition of viral RNA sunthesis	King et al. (2002)
Thiosemicarbazone derivatives	Thiosemicarbazone family	Demonstration of efficacy	Finkielsztein et al. (2008)
Thiosemicarbazone	Thiosemicarbazone family	Inhibition of replication complexes	Castro et al. (2011)

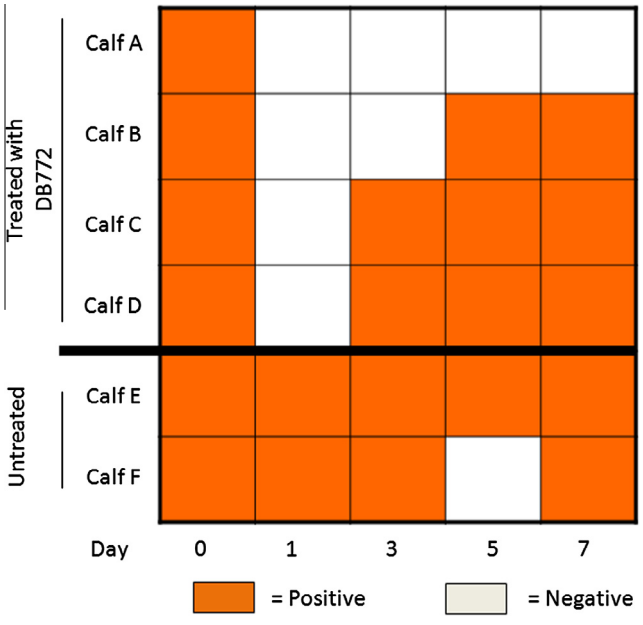

Fig. 4. Virus isolation (VI) of passaged white blood cells from calves persistently infected with bovine viral diarrhea virus. Calves A–D were treated with the antiviral compound DB772 beginning on Day 0 and continuing through Day 6. All treated calves were VI negative after treatment initiation; mutant isolates were isolated from calves B, C and D on Days 5, 3 and 3, respectively (Newcomer et al., 2012a).

(siRNA) or short hairpin RNA (shRNA) (Pratt and MacRae, 2009). Due to the specific nature of antisense therapies, identifying conserved regions of the genome is of the utmost importance to provide the broadest range of protection from designed oligonucleotide sequences. Antisense therapy has proven effective at inhibiting BVDV in cell culture when using siRNA and shRNA targeting the 5́ UTR and certain conserved structural (Mishra et al., 2011)or nonstructural (Lambeth et al., 2007) regions or a combination of both (Ni et al., 2012). The specific nature of antisense strategies increases their potential for use as targeted antiviral therapies. However, antisense applications for veterinary medicine are currently experimental and will require significant additional characterization before commercial implementation is possible.

2.4.3. Results of in vivo testing

There are few reports of *in vivo* testing of antiviral compounds against BVDV. In the first published report of the treatment of PI calves with a specific antiviral compound (DB772), we demonstrated markedly reduced viremia in treated calves (Fig. 4) (Newcomer et al., 2012a). Previously, DB772 and related aromatic cationic molecules had been used to eliminate BVDV infection in contaminated bovine fetal fibroblast cells, infected cell lines and embryo culture media (Givens et al., 2004, 2005). Furthermore, bovine embryos obtained from *in vitro* fertilization (IVF) that were cultured with the compound were successfully implanted into recipients and yielded healthy calves with normal reproductive capacity (Givens et al., 2006, 2009). In our recent study, intravenous administration of the compound to PI calves transiently

decreased viral titers in all treated calves to below levels detectable by virus isolation techniques, but resulted in the rapid selection of resistant isolates (Newcomer et al., 2012a). The current recommendation for PI animals is to identify and remove them from the herd to limit virus transmission. We do not disagree with this recommendation, but propose that the PI animal represents the most robust *in vivo* test of a novel antiviral compound because of the consistent high level of viremia exhibited by affected animals.

In a second pilot study, administration of DB772 before intranasal challenge with BVDV successfully prevented infection in seronegative calves, but left them susceptible to infection after protective levels of the compound had waned (Newcomer et al., 2013a). Further study must be directed at alleviating concerns of renal toxicity raised in the second study and developing a more user-friendly formulation practical for wide-scale commercial use (Newcomer et al., 2013b). With further development, the antiviral properties of DB772 and related compounds could potentially be safely employed in multiple situations to treat or prevent BVDV infections.

Interferons are multifunctional proteins produced and released from host cells in response to the presence of pathogens, particularly viruses. Interferons of both human (HuIFN) and bovine (BoIFN) origin have been widely studied as nonspecific antiviral compounds in relation to BVDV. Both CP and NCP biotypes of BVDV are considered highly susceptible to *in vitro* interferon treatment (Peek et al., 2004a). However, the value of IFN administration to treat or prevent BVDV infection in the live animal has been difficult to demonstrate. Viral load in PI cattle decreased slightly after subcutaneous treatment with BoIFN-τ when animals were treated with 10^6 U/kg ten times over the course of a two-week period (Kohara et al., 2012). A similar decrease was not seen when cattle were treated with only 10^5 U/kg or with oral or subcutaneous administration of HuIFN-α (Kohara et al., 2009). However, decreases in viral titer seen with administration of the higher dose of BoIFN-τ were transient and were erased following cessation of treatment.

In an extended study, in which five Holstein PI heifers were treated with recombinant human IFN-α every other day for 84 days, no antiviral activity of the treatment could be demonstrated (Peek et al., 2004b). Furthermore, the treated heifers all developed a microcytic anemia during the treatment period, secondary to the production of anti-interferon antibodies. Although *in vitro* studies have demonstrated good proof of concept, the poor *in vivo* efficacy and need for extended treatment periods limit the practical application of IFN therapy to curtail BVD.

2.4.4. Potential role of antiviral drugs in BVDV control

The value of using antiviral therapies to treat PI animals remains to be determined. The role of such animals in disease transmission has led to the current recommendation of identification and culling. The combination of the low prevalence of PI animals and their high level of viremia makes treatment challenging and cost-prohibitive. However, the consistent viremia exhibited by PI animals makes them an ideal and very stringent *in vivo* test for novel antiviral compounds (Newcomer et al., 2012a). Consequently, the applied use of antivirals in BVDV control will likely be for the prevention, rather than the treatment of persistent infections.

In areas of endemic BVD, the expense and labor of antiviral administration will be difficult to justify in individual commercial cattle experiencing transient infection due to the low mortality and often subtle clinical signs of infection. In such regions, the economic return will be greater for expenditures on preventive measures such as vaccination or biosecurity programs than for the treatment of infected animals. However, strategic use of antivirals in endemic areas could be employed for the protection and preservation of valuable breeding stock, rare zoological collections and endangered species against BVDV (Goris et al., 2008). Evidence of infection has been documented in over 50 families within the order *Artiodactyla*, which also contains several threatened and endangered species (Passler and Walz, 2010). Currently, no specific therapies exist for the protection of valuable or rare hoofstock, and while most infections result in low mortality, outbreaks of severe morbidity and high mortality have been reported (Pellerin et al., 1994; Hessman et al., 2012).

The use of prophylactic antiviral agents in veterinary medicine is still in the developmental stage, but the possibility of using such compounds in the control of BVD is attractive. Effective antiviral agents have the advantage of providing near-immediate protection from viral challenge. In a recent study, we showed that prophylactic administration of an antiviral compound to seronegative calves protected from experimental challenge less than 24 h following the initial administration of the compound (Newcomer et al., 2013a). The benefits of such a compound would be particularly useful in a feedlot situation where calves of unknown immune status are commingled and exposed to a variety of pathogens. The estimated prevalence of PI animals entering the feedlot is 0.3% (Loneragan et al., 2005); therefore naïve calves will likely be exposed to BVDV soon after arrival. While calves are often vaccinated soon after arrival, the delay in immune protection provides a window for viral exposure to result in infection and disease. The use of specific antiviral therapy could provide adequate protection until vaccine protection is realized, but the effect of simultaneous antiviral therapy and MLV vaccination has yet to be studied.

BVD has been eliminated from some regions, and campaigns are ongoing in others to eradicate the virus (Ridpath, 2010b). Many regions no longer allow vaccination due to the difficulty in distinguishing vaccinal and natural exposure in serologic assays, leaving the cattle population susceptible to BVDV. In outbreaks of disease among cattle with large numbers of susceptible animals, prophylactic administration of effective compounds could be used to limit viral transmission and subsequent animal losses. Additionally, administration of antiviral drugs does not complicate diagnostic efforts in treated animals, allowing unhindered disease monitoring for continued control. By preventing infection, antiviral administration averts a serologic response to viral challenge in treated animals. Such animals would still be capable of mounting an immune response to subsequent challenge after protective levels of the compound have waned, allowing continued surveillance.

The potential for the control of BVDV through the use of antiviral drugs is bright but as yet, unrealized. The lack of demonstrated *in vivo* efficacy of identified compounds is currently the biggest hurdle to their commercial implementation. Additional study must focus on practical methods to deliver effective drugs. The availability of such compounds would be invaluable in the control of disease outbreaks by providing immediate directed protection from the virus.

3. Classical swine fever

3.1. Clinical signs and transmission

3.1.1. Transient infection

CSF tends to be more severe and result in higher mortality than BVD. Historically, the disease has occurred in epidemics, with morbidity and case fatality rates approaching 100%; more recently, less virulent strains have caused slower-spreading epidemics, with milder clinical signs (Floegel-Niesmann et al., 2003). Highly virulent isolates cause severe acute disease. Pigs may rarely be found dead without prior signs of illness. More commonly, lesions are suggestive of generalized septicemic disease and are preceded by high fever (Lohse et al., 2012). Affected pigs are depressed,

anorexic and often huddle together for warmth. Multiple hemorrhages are evident on the skin and exudative conjunctivitis can be severe (Everett et al., 2010). Constipation is followed by severe diarrhea. Neurologic signs often accompany disease caused by isolates of high virulence, beginning as circling, incoordination and muscle tremors before progressing to convulsions shortly before death. Disease spreads rapidly though affected herds and deaths can be expected from 5 to 15 days after clinical signs are first seen. Syndromes of subacute illness are characterized by milder clinical signs, with deaths occurring approximately one month after of the first clinical signs.

A chronic disease syndrome has been described that occurs following infection with low virulence strains (Hulst et al., 2013). Pigs present with anorexia, persistent fever, depression and skin hemorrhages after a prolonged incubation period. Affected pigs seem to recover and appear clinically normal, but may relapse and die if stressed, though the mortality rate is much lower than for more virulent strains. Chronically infected pigs fail to thrive and appear more susceptible to coinfection with bacterial pathogens.

3.1.2. Persistent infection

As with BVDV and BDV, congenital infection with CSFV may result in abortion, malformations and the birth of PI offspring (Dewulf et al., 2001). Infections in the first trimester of gestation most often result in fetal loss, whereas infection between days 50–70 of gestation may result in the birth of PI piglets. While an important reservoir of the disease, the PI animal is less central to the transmission of CSFV than of BVDV. The presence of chronic infections serve as an additional viral reservoir, and the increased severity of clinical signs often results in earlier detection of PI animals. In addition to persistent infections, prenatal infection may also result in malformations including cerebellar hypoplasia, hypomyelogenesis and skeletal deformities. Despite appearing clinically normal for up to several months, PI pigs may develop "late onset" disease characterized by anorexia, depression, stunted growth, diarrhea and occasionally congenital tremors (Terpstra and Wensvoort, 1997). Death is consistently seen before one year of age.

3.1.3. Transmission

Infected swine serve as the reservoir of CSFV. The wild boar has been the source of infection for several primary outbreaks of the disease in Europe and remains a major risk factor for new outbreaks (Fritzemeier et al., 2000; Boklund et al., 2008). Transmission to susceptible swine occurs most commonly through direct contact by ingestion of infected material. As with BVDV, CSFV is shed in all bodily secretions from infected animals. Aerosol transmission is limited to distances of one meter or less (Gonzalez et al., 2001). Mechanical transmission through contaminated equipment is possible, but not a primary means of viral spread in most outbreaks. Ingestion of contaminated pork containing infectious virus is a possible source of infection (Edwards, 2000). Artificial insemination using infected semen can infect susceptible sows and gilts. However, the most common route of transmission remains direct contact with other infected swine.

3.2. Diagnosis

Detection of viral antigen is recommended for the rapid confirmation of CSF in infected pigs. The direct immunofluorescence antibody technique is rapid, economical and sensitive and formed the primary test used during the eradication campaign in the United States. The assay can detect infection as early as two days after infection in tonsillar material, allowing prompt identification and elimination of infected animals. Other samples of choice include the spleen, ileum and lymph nodes. The utilization of CSFV-specific monoclonal antibodies is recommended to avoid false-positive results that may be seen in pigs infected with BVDV when polyclonal antibodies are used. The highest diagnostic sensitivity of the test is seen during acute infection. Antigen-capture ELISAs have also been developed for the rapid screening of large numbers of pigs with clinical suspicion of CSFV infection.

As with BVD, virus culture techniques have historically been considered to be the most sensitive diagnostic test for CSF. Leucocytes are often considered the best sample from which to culture virus although in the early phase of infection, isolation from whole blood or plasma is more rewarding than from the buffy coat (Gisler et al., 1999). Virus may also be isolated from tissue samples. The high sensitivity of virus isolation is important to correctly identify infected pigs but virus isolation techniques are labor-intensive and time consuming. The sensitivity of RT-PCR is comparable to or exceeds that of virus isolation and has a much shorter turn-around time. Early RT-PCR assays used pan-pestivirus primers from the 5' untranslated region, but more recently developed tests are capable of differentiating between CSFV and BVDV using different regions of the genome (Zhang et al., 2012).

Another important feature of the RT-PCR assay is the ability to sequence outbreak isolates to assist in epidemiologic investigations.

Antibody detection techniques are easily employed to screen pig herds for evidence of previous CSFV infection. These techniques have been widely used in eradication efforts as well as in disease free regions to monitor for inapparent infections. Antibodies to the ruminant pestiviruses may cross-react with those of CSFV; further testing (e.g., virus neutralization testing) may therefore be necessary to differentiate the type of infection although CSFV-specific assays are available. The main drawback to the use of serological techniques for the diagnosis of CSFV is the inability to discriminate between animals vaccinated with conventional attenuated vaccines. Consequently, significant incentive exists to develop marker vaccines that will allow the continued use of serologic assays without compromising their diagnostic integrity.

3.3. Vaccines

3.3.1. Role of vaccines in the control of CSF

The role of vaccines in the control of CSFV transmission is complicated by international trade regulations and the need for highly effective vaccines which adhere to DIVA principles. In countries where the disease has been eradicated, or at least cleared from the domestic swine population, vaccination is generally prohibited, so as to allow detection of virus outbreaks and maintain established trade requirements. In endemic areas, vaccination is an effective means to limit transmission of the virus, prevent disease outbreaks and establish protective immunity in naïve swine populations. Conventional vaccines are not DIVA-adherent (see below), and consequently they are often not used in previously disease-free areas experiencing CSF outbreaks. Instead, massive culling of infected and at-risk animals is often undertaken in order to more quickly reestablish the ability to export swine and pork products. The development of a vaccine that provides effective immunity against acute and fetal infection and adheres to the principles of DIVA is crucial for use in such situations to limit the culling of non-infected animals.

3.3.2. C-strain vaccine

The MLV, C-strain CSF vaccine is safe and effective for use in endemic areas and in outbreak control (as reviewed by van Oirschot (2003)). The vaccine provides complete protection in as little as five days against highly divergent viral genotypes, with evidence of partial protection and decreased transmission seen as early as one day after vaccination (Graham et al., 2012a). Clinical protection is afforded by stimulation of both the humoral and

cell-mediated arms of the immune system (Graham et al., 2012b). Oral immunization also appears to prevent fetal infection in challenged gilts, preventing the creation of PI piglets (Kaden et al., 2008).

Bait vaccines based on the C-strain of the virus have proven safe and effective in the oral immunization of wild boar (von Ruden et al., 2008; Kaden et al., 2010). Consequently, the use of the C-strain vaccine has been widely used in CSF eradication and containment efforts. However, use of the vaccine does not allow DIVA which can lead to prolonged trade restrictions between countries where the disease has been eradicated. This has prompted the use of depopulation and pre-emptive culling as the main tools to curtail exotic CSF outbreaks. Consequently, pressure has mounted to develop a vaccine adherent to the DIVA principle with equitable safety and efficacy as conventional vaccines. The development of suitable marker CSF vaccines will be imperative to controlling the disease while maintaining the integrity of trade regulations with the current diagnostic infrastructure.

3.3.3. Subunit vaccines

Subunit vaccines are the most advanced DIVA vaccination strategies, and are based on the immunogenic E^{rns} and E2 glycoproteins. While the E^{rns} vaccines have failed to demonstrate a protective effect against CSFV challenge (Lin et al., 2012), two vaccines based on baculovirus-expressed E2 glycoprotein were previously marketed commercially in Europe. Vaccinated pigs develop antibodies exclusively to the E2 protein, whereas naturally infected animals also develop antibodies to the E^{rns} envelope protein, thus allowing detection of vaccinated animals (de Smit et al., 2001a). However, as antibodies to the E^{rns} protein may not be present early in infection, caution must be taken when interpreting results. More importantly, subunit vaccines offer incomplete protection compared with conventional MLV vaccines, and are no longer commercially available. Lapses in protection of pregnant sows are particularly concerning, as this results in a higher risk of the establishment of PI pigs which might serve as a reservoir for maintenance of CSFV in vaccinated herds (Ahrens et al., 2000; de Smit et al., 2000; Dewulf et al., 2005).

A second concern with subunit vaccines that may limit their use in containing outbreaks has been a delay before the onset of complete protection (Bouma et al., 1999). Early onset is crucial to minimize transmission to vaccinates in disease outbreaks. An experimental vaccine produced in the milk of adenovirus-transduced goats appears to provide protection from clinical signs and viremia within one week after vaccination (Barrera et al., 2010; Toledo et al., 2010). Its efficacy in pregnant animals remains to be assessed. For subunit vaccines to be implemented on a wide scale, their level and time of onset of protection must be increased to make them comparable to conventional vaccines.

3.3.4. Chimeric vaccine strategies

The most promising experimental DIVA vaccination strategy appears to be the use of recombinant chimeric vaccines. With this strategy, the potential benefits of live vaccination, including early onset of protection and long duration of immunity, are coupled with DIVA capability. Attenuated viral vectors, nonpathogenic to the host, are used to deliver target antigens (E^{rns} or E2) which are then constitutively expressed in vaccinates. Chimeric pestiviruses in which either the E^{rns} or E2-encoding regions of CSFV incorporated into a cDNA copy of another pestivirus, or vice versa, have been the most successful candidates for a marker CSFV vaccine. Replacement of the antigenic portion of the E2 gene or the E^{rns} gene of the CSFV C-strain with the homologous BVDV regions resulted in a recoverable virus that induced specific antibody responses that could be differentiated from natural infection (van Gennip et al., 2000). Inoculated pigs were completely protected against a lethal CSFV challenge. In a follow-up study, transmission to specific-pathogen-free pigs was not seen after challenge of pigs vaccinated once intramuscularly with the chimeric vaccine (de Smit et al., 2001b). Likewise, replacing the E2 gene of CSFV with the corresponding sequence from BDV resulted in a marker vaccine that gave full protection from challenge after intramuscular injection and partial protection following oral vaccination (Wehrle et al., 2007).

Reimann et al. (2004) used a BVDV vector to express the CSFV E2 protein. The chimeric pestivirus, CP7_E2alf, has been extensively studied and represents the most advanced live vaccine marker candidate to date. Protection is comparable to that seen with conventional C-strain vaccines following intramuscular, oronasal or oral administration (Reimann et al., 2004; Tignon et al., 2010; Renson et al., 2013; Eble et al., 2013), while adhering to the DIVA principle (Leifer et al., 2009a; Aebischer et al., 2013). Clinical protection is not hindered by the presence of maternal antibodies at the time of vaccination (Rangelova et al., 2012) and persists for at least 6 months following oral or intramuscular administration (Gabriel et al., 2012). Full clinical protection was also demonstrated one week after intramuscular injection and two weeks after oral administration, making CP7_E2alf a potential candidate for emergency vaccination in a disease outbreak (Leifer et al., 2009b).

Despite the potential of CP7_E2alf as a marker vaccine for CSF, questions must still be addressed regarding the safety and licensure of genetically modified vector vaccines. Initial studies indicate that CP7_E2alf is safe in calves, kids, lambs and rabbits (Konig et al., 2011). Continued study and development of CP7_E2alf leading to licensure would provide an additional tool for CSF outbreak control and eradication efforts, by providing efficient prophylaxis while allowing DIVA.

3.4. Antiviral drugs

3.4.1. Results of in vitro testing

Unlike BVDV, CSFV has not commonly been used to screen potential antiviral compounds for evidence of efficacy, and documented evidence even of *in vitro* activity is therefore scarce (Freitas et al., 2003; Krol et al., 2010). However, given the similarity of the genomic organization and protein products of the two viruses, compounds with efficacy against BVDV are likely to also be effective against CSFV. Various compounds have been shown to be active against multiple pestiviruses (Paeshuyse et al., 2006; Newcomer et al., 2012b), and the identification of effective compounds against one agent therefore warrants testing against the others. For practical implementation, however, different drug formulations may be necessary, particularly for oral administration, as swine are monogastrics, while cattle and sheep are ruminants. Alternative antiviral therapies for CSF control, including capsid-targeted virus inactivation (Zhou et al., 2010; Wang et al., 2010), RNA interference (Porntrakulpipat et al., 2010) and the use of RNA-hydrolyzing recombinant antibody (Jun et al., 2010) have been explored in cultured cells, but their practical implementation will require significant additional study and development.

3.4.2. Results of in vivo testing

Only one drug, the imidazopyridine BPIP, has documented evidence of *in vivo* activity against CSFV. It exhibits potent *in vitro* antiviral activity against several pestiviruses through inhibition of the RdRP, and is the most thoroughly studied antiviral compound against the pestiviruses (Paeshuyse et al., 2006; Vrancken et al., 2008; Haegeman et al., 2013). Although it did not completely prevent infection, piglets fed BPIP for 15 consecutive days at a daily dose of 75 mg/kg, beginning one day before challenge with CSFV, had significantly reduced mean viral genome loads, duration of viremia and mean CSFV antibody titers, compared to untreated

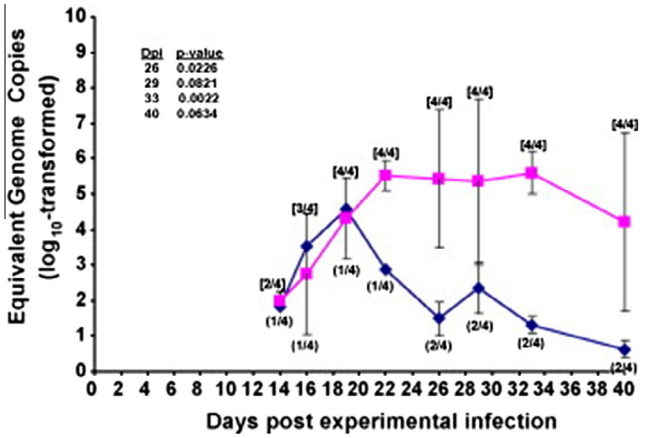

Fig. 5. Mean virus genome load in blood of untreated sentinel animals in contact with BPIP-treated (diamonds; $n = 4$) and untreated (squares; $n = 4$) seeder pigs as determined by means of real-time RT-PCR. Number of animals tested positive at a given time point is shown between brackets. Statistical significance (p values) is given at each measurable timepoint. Figure reprinted with permission (Vrancken et al., 2009a).

cohorts (Vrancken et al., 2009b). The severity of clinical signs in the control group resulted in the euthanasia of 75% of the untreated piglets, while all treated piglets survived until the study's endpoint. Infectious virus also could not be isolated from the tonsils of treated pigs at the study's endpoint.

Administration of BPIP in feed would allow for easy dosing of large numbers of swine, with minimal labor inputs. In an outbreak scenario, this would provide protection to surrounding herds promptly and efficiently. A follow-up pilot study involving four BPIP-treated pigs revealed a 50% reduction in CSFV transmission to untreated sentinel pigs (Fig. 5) compared to untreated controls (Vrancken et al., 2009a). The use of BPIP or similar compounds in CSF outbreaks has the real potential to decrease the spread of the virus and sharply reduce animal and economic losses (Backer et al., 2013).

3.4.3. Potential role of antiviral drugs in CSF control

Due to the high level of international regulatory control of CSF, there is great pressure to rapidly control outbreaks regions from which it has been eradicated, including North America and Europe. The current outbreak control strategy is tied to "stamping out" policies, which involve the depopulation of affected herds and at-risk animals. Emergency vaccination strategies are often not pursued, due to the inability to differentiate vaccinated from infected animals (DIVA) using common serological assays. Vaccination strategies are also limited by the delay in protection provided by current vaccines. The prophylactic implementation of effective antiviral therapy therefore has the potential to improve animal welfare, minimize disruption of trade and decrease the economic impact of CSF.

In a stochastic modeling study comparing the use of prophylactic antiviral supplementation to depopulation, preemptive culling and/or emergency vaccination, the use of antivirals performed equally as well or better than any other proposed containment measure or their combination (Ribbens et al., 2012; Backer et al., 2013). The ability of antiviral compounds to provide almost immediate protection from viral challenge makes them extremely attractive alternatives for outbreak control (Haegeman et al., 2013), but the large-scale application of antiviral prophylaxis will ultimately depend on the cost and effectiveness of the available compound. Ease of storage and administration will also be crucial for the rapid implementation of antiviral protection (Goris et al., 2008). Given the public's increasing distaste for the pre-emptive depopulation of unaffected herds, the potential for antiviral prophylaxis is increasingly attractive.

The application of antiviral strategies for the control of CSF will likely be limited to preventive, as opposed to therapeutic modalities. In areas free of the disease, treatment of even extremely valuable animals is hard to justify, given the potential for chronic infection seen after infection with certain viral strains (Hulst et al., 2013). Even in endemic areas, the treatment of valuable breeding stock poses the concern of vertical transmission if the treatment is not fully effective. However, in those areas where affected animals are not culled for the sake of halting transmission, antiviral therapy of infected individuals could limit the severity of clinical signs and speed healing. Even so, the prophylactic application of an antiviral is more attractive than therapeutic applications, given the regulatory concern surrounding CSF.

4. Border disease virus and the atypical pestiviruses

4.1. Clinical syndrome

4.1.1. Transient infection

Border disease virus is the third official member of the *Pestivirus* genus. Border disease is found worldwide and primarily affects sheep, although goats are occasionally infected. Clinical disease is largely limited to lambs infected *in utero*, and is similar to what is seen in cattle PI with BVDV. With few exceptions, infection of adolescent or mature sheep results in subclinical viremia, without gross or microscopic lesions. As with BVDV, fetal infection causes most of the clinical signs, and the disease syndrome is largely determined by the age of the fetus at the time of infection (Roeder et al., 1987). Abortion is most common in the first trimester of gestation and may go unnoticed as the ewe often does not show signs of illness. Fetal loss during the first trimester may be due to direct infection of the fetus or may be secondary to an acute necrotizing placentitis (Barlow and Patterson, 1982).

4.1.2. Persistent infection

Fetal infection between 21 and 72 days of gestation may result in PI lambs, in which signs of disease are most often seen (Roeder et al., 1987). As with calves PI with BVDV, PI lambs are more susceptible to secondary disease and have higher overall mortality rates, but may be phenotypically normal. Such animals are viremic throughout their lives and constantly excrete infectious virus. "Hairy shaker" is the colloquial term for PI lambs, due to their typical neurologic and fleece abnormalities. In affected animals, primary hair follicle enlargement, coupled with a decrease in the number of secondary hair follicles, results in large medullated primary fibers. Hypomyelinogenesis results in neurologic dysfunction, ranging from fine tremors to tonic-clonic contractions of the body and head during the first 6 months of life (Jeffrey and Roeder, 1987). Other clinical signs of PI lambs include poor growth rate, delayed onset of puberty, skeletal deformities, behavioral and visual defects and impaired reproductive performance (Garcia-Perez et al., 2009).

4.2. Diagnosis

Although more advanced than the diagnostics for the atypical pestiviruses, specific assays for the detection of BDV are less well developed than those for BVDV and CSFV. The most sensitive way to confirm BDV viremia is to isolate infectious virus from washed leukocytes. Spleen, thymus, thyroid, lymph nodes, brain and kidney represent the best organ samples for virus isolation in the dead animal. However, virus isolation is expensive and time consuming when used as a herd screening tool. Consequently,

commercial ELISA kits have been developed to detect antigen in PI sheep using blood or bulk-tank milk samples (Corbiere et al., 2012). However, the tests are usually not sufficiently sensitive to detect acutely viremic animals. Antigen-capture ELISAs used for detecting BVDV that recognize structural protein epitopes are unreliable for the detection of BDV. As with BVDV and CSFV, nucleic acid detection methods have gained widespread popularity for diagnosing infection with BDV. Primers specific to BDV have been identified that can be used in a one-step PCR or following the use of pan-pestivirus primers in a nested PCR reaction (Willoughby et al., 2006). Serological diagnostic tools for BDV include the virus neutralization assay, ELISA and the less sensitive agar gel immunodiffusion.

4.3. Role of vaccines in border disease control

There are few vaccines for border disease, and they face many of the same challenges seen with BVD and CSF. Inactivated vaccines have been developed for use in sheep but are unlikely to stimulate long lasting immunity, requiring regular boosters to maintain an adequate level of protection against field challenge (Vantsis et al., 1980; Brun et al., 1993). Isolates of BDV can be separated into at least three distinct genotypes (Becher et al., 2003). Effective vaccines must therefore be formulated from viral strains exhibiting a wide degree of cross-protectivity, or must incorporate several viral strains. Ideally, BDV vaccines would also provide some degree of protection from BVDV, as both viruses commonly infect sheep. Effective BDV vaccines must prevent transplacental spread of the virus, but current information is not sufficient to determine the level of vaccinal antibody titers necessary to protect the fetus.

4.4. Potential role of antiviral drugs in BDV control

Antiviral drugs against BDV remain largely unexplored. Phylogenetic analysis reveals a very close relationship between CSFV and BDV isolates, with increasing diversity seen in isolates of the atypical pestiviruses (Kirkland et al., 2007). Consequently, antiviral targets identified in BVDV and CSFV may also be suitable for antiviral therapy against the remaining pestiviruses, as suggested by the few published studies exploring antiviral drug therapy in BDV. Thus, a compound with demonstrated *in vitro* and *in vivo* efficacy against BVDV was found to exhibit similar or enhanced levels of efficacy *in vitro* against BDV (Newcomer et al., 2012b). BDV replication is also inhibited *in vitro* by BPIP (Paeshuyse et al., 2006) and by an imidazopyrrolopyridine analogue (Paeshuyse et al., 2007), both of which are effective against BVDV and CSFV. Because economic losses and regulatory concerns are much higher for CSF and BVD than for border disease, there is little impetus to test antiviral drugs for this disease. In the absence of specific studies with BDV, information gleaned from antiviral studies employing BVDV and CSFV should be extrapolated with caution.

5. Directions for future research

The potential use of antiviral therapy to control pestiviral disease is promising, but in the case of most compounds, further development will be needed before field applicability can be realized. Many compounds are effective at inhibiting BVDV *in vitro*, but their efficacy must be demonstrated in live animals under controlled conditions before field trials can be attempted. Licensed antiviral compounds effective against BVDV, CSFV or BDV are therefore unlikely to become available in the foreseeable future, with the possible exception of the few compounds that have already been tested *in vivo*. In the case of CSFV, the administration of BPIP to pigs in their feed appears to be effective at decreasing viral transmission (Vrancken et al., 2009a); it may therefore be the leading candidate for commercial development. We have demonstrated the antiviral efficacy of DB772 in the prevention and treatment of BVDV infections, but the current formulation is not practical for field use, and concern about potential negative side-effects must be addressed before the drug could be employed commercially (Newcomer et al., 2013a). Although their potential to provide instantaneous protection makes antiviral drugs highly attractive candidates for the control of pestivirus outbreaks, *in vivo* data are currently lacking for most drug candidates.

In our opinion, the use of antiviral therapy for the control of pestiviral disease will be most effectively utilized to contain outbreaks. Effective compounds provide rapid protection against virus challenge, and antivirals may therefore be initiated at the time of outbreak recognition, without the need for continued, prolonged prophylactic administration in the absence of a recognized and defined outbreak. The timely use of an effective drug has the potential to limit the widespread culling of at-risk and unexposed animals, which is often undertaken in an effort to curtail outbreaks and minimize trade restrictions. Future research should therefore be focused on identifying and developing highly effective compounds that can be quickly and easily administered to large numbers of animals. The pharmacokinetics of such drugs must also be studied, to establish accurate withdrawal times for milk and meat products, to avoid introducing drug residues into the human food chain.

A highly effective marker vaccine that can reliably distinguish vaccinates from naturally exposed animals using serological techniques will be crucial to the implementation of emergency vaccination as a means of outbreak control, particularly for CSF. While conventional vaccines are still used to effectively control CSF in endemic areas, they are prohibited in regions free of the disease, in order to maintain the current diagnostic infrastructure. While several strategies are currently being studied, vaccines based on chimeric pestiviruses appear to be most promising. The CP7_E2alf is the most thoroughly studied chimeric vaccine, and initial trials indicate acceptable efficacy and safety. However, questions regarding the licensure of a genetically modified vector vaccine must be addressed before it can be employed commercially.

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