

Prevalence of Bovine viral diarrhea virus subspecies among persistently infected positive samples submitted to a diagnostic laboratory from cattle in the United States.

by

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Abstract

Bovine viral diarrhea virus (BVDV) is an infectious disease affecting ruminants worldwide. Impacting the respiratory, reproductive, and digestive systems, BVDV remains one of the most economically damaging diseases to cattle producers. Previous phylogenetic analysis has divided the virus into two species, BVDV1 and BVDV2, with three main subspecies circulating in U.S. cattle populations: BVDV1a, BVDV1b, and BVDV2a. The objective of this study was to determine the prevalence of the three subspecies in cattle across the United States. Samples were obtained from various segments of the industry: cow/calf, stocker, feedlot, and dairy. Samples used were from live animals where fresh skin (ear notch) had previously tested positive for persistent infection via antigen capture ELISA (ACE) or immunohistochemistry (IHC). This study was comprised of 1,093 samples from 21 states, with a majority of samples from Kansas, Kentucky, Oklahoma, and Texas. Positive samples were submitted to a university diagnostic laboratory and segregated into three subspecies (BVDV1a, BVDV1b, and BVDV2a) via Reverse-Transcriptase PCR (RT-PCR) by sequencing of the 5'-untranslated region (5'-UTR). 1,000/1,093 samples were confirmed positive by PCR. Of the PCR confirmed samples, the prevalence of subspecies BVDV1b BVDV1a, and BVDV2a was 702/1,000 (70.2%), 44/1000 (4.4%), 178/1000 (17.8%), respectively, with 76/1000 (7.6%) of samples unable to be translated successfully. These findings support previous studies exhibiting BVDV1b as the most predominant subspecies among cattle, persistently infected with bovine viral diarrhea virus, in the United States.

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Chapter 1 - Review of Literature

Bovine Viral Diarrhea Virus (BVDV)

Bovine viral diarrhea virus (BVDV) is a member of the *Pestivirus* genus within the family Flaviviridae (Ridpath, 1994). Other notable members of the *Pestivirus* genus include classical swine fever virus (CSFV) and border disease virus (BDV), infecting pigs and sheep, respectively (Omari et al., 2013). BVDV viral particles have a diameter of 40-60 nm and consist of a pleomorphic outer lipid envelope surrounding an inner protein shell or capsid (Ridpath, 2010). BVDV is a positive-sense single-stranded RNA virus with a 12.5 kb genome that contains a single, large open reading frame (ORF) encoding a single polyprotein, cleaved by viral and cellular proteases into 12 structural and non-structural proteins (Lindenbach and Rice, 2001). Four structural proteins (SPs), a basic core protein C and the envelope (E) glycoproteins Erns, E1, and E2, are found in the virion. The E surface proteins are inserted into the viral envelope which is derived from intracellular membranes of the host cell (Lindenbach et al., 2013). Both proteins, Erns and E2 are implicated in blocking the host antiviral defense (Tautz et al., 2015). The ORF is preceded and followed by 5' and 3' untranslated regions (UTR) of 360 to 390 nucleotides and 200 to 240 nucleotides, respectively. Much of the heterogeneity observed among BVDV is caused by variability inherent in having a single-stranded RNA genome. Mutations easily occur in each replication as there is no proofreading function associated with replication (Ridpath, 2010).

Isolates of bovine viral diarrhea virus were further segregated into two species, BVDV 1 and BVDV 2 based on comparisons of sequences of from the 5' untranslated region. Phylogenetic analysis suggested that the two are as different from each other as reference BVDV strains were from classical swine fever (Ridpath 1994). BVDV type 1 and type 2, have a

nucleotide sequence homology of roughly 60%. More recently, BVDV1 has been divided into at least 21 subspecies, with BVDV1a and BVDV1b being the predominant subspecies, and BVDV2 into 3 subspecies, BVDV1a, BVDV2b and BVDV2c. The newly BVDV2b subspecies was previously recognized only in Asia, as the few U.S isolates previously identified as BVDV2b were re-classified as BVDV2c after further investigation (Neil et al., 2019).

Both BVDV1 and BVDV2 viruses may exist as one of two biotypes, cytopathic (CP) and non-cytopathic (NCP), with non-cytopathic BVDV predominating in nature. The reason for the high prevalence of the NCP biotype in the field is its ability to establish persistence upon fetal infection leading to constant virus shedding by persistently infected animals (Tautz et al., 2015), as described later. Ridpath summarized that proteins associated with BVDV replication in cultured cells reveal that cytopathic BVDV could be distinguished from noncytopathic BVDV by the production of an extra nonstructural protein known as NS3, the result of the cleavage of another nonstructural protein, NS2/3. Comparison of NS2/3 coding region of cytopathic and non-cytopathic BVDV revealed that genomes of most cytopathic viruses are the product of genetic recombination(Ridpath, 2010). Cells infected by pestiviruses of the CP biotype swell up, detach from the monolayer, and die, most likely, due to the induction of apoptosis. Conversely, replication of NCP viruses does not lead to microscopically detectable alterations in cells (Tautz et al., 2015). The cp virus is the trigger for the switch from the long-lasting persistent infection(PI) to lethal Mucosal Disease MD which was finally established by the superinfection of PI animals with CP BVDV (Bolin et al., 1985), described later.

Acute Infection of BVDV

BVDV can induce very different symptoms in the host animals (Baker, 1995). Acute (transient) BVDV infection is the term used to describe clinical or subclinical disease that occurs

in non-persistently infected immunocompetent cattle following exposure to BVDV (Ridpath, 2010) The initial description of BVDV was an acute enteric disease of cattle, characterized by outbreaks of diarrhea and erosive lesions of the digestive tract, first reported from North America in 1946 (Childs, 1946). Exposure is typically from short range, large droplet aerosols, or through direct contact with infected animals. The infection spreads from the nasal mucosa to the draining lymph nodes and from there is transmitted to other tissues via circulating lymphoid cells. Symptoms associated with BVDV infection may be transient leukopenia, mild fever, diarrhea, increased nasal discharge, coughing, and other signs of abnormal respiration. Transiently infected cattle are much less efficient transmitters of the virus than persistently infected animals. They secrete low levels of virus for an average of 12 days versus PI individuals that shed copious amounts of virus for the entirety of life (Larson, 2015). Virus infection has an incubation period of 5 to 7 days and viremia is typically less than 15 days (Ridpath, 2010). When immunocompetent cattle are exposed to the BVD virus, the result of the majority of these infections is subclinical in nature. It has been estimated that 70% to 90% of BVDV infections occur without manifestation of clinical signs (Ames, 1986).

A hemorrhagic syndrome has also been reported from BVDV infection. This syndrome is characterized by marked thrombocytopenia, which results in bloody diarrhea, epistaxis, petechial and ecchymotic hemorrhages on mucous membranes, and bleeding from injection sites. The hemorrhagic syndrome associated with BVDV infection has been associated with noncytopathic isolates of BVD (Moennig, 2005). The BVD viruses responsible for this condition were the first recognized members of the BVDV2 species (Tautz et al., 2015). A severe BVDV2a outbreak in Canada described clinical signs of pyrexia, pneumonia, and diarrhea in all age groups. Oral ulcers were frequently observed in older cattle. Abortions also were reported in association with

these clinically severe BVD outbreaks (Pellerin, 1994). Many of the clinical signs are similar for type 1 and type 2 BVDV. One difference for BVDV2 is the development of thrombocytopenia during an infection (Nagele, 1984). Additionally, non-cytopathic BVDV strains are more commonly associated with thrombocytopenia.

Another acute clinical sign of BVDV is known as Mucosal Disease (MD), which occurs when cattle that are persistently infected with a non-cytopathic BVDV become superinfected with a cytopathic BVDV (Baker, 1995). The syndrome develops when a cytopathic BVDV, often an escape mutant from the animal's own noncytopathic BVDV from the fetal infection, is antigenically similar to the noncytopathic internal BVDV. Onset of MD is sporadic and most often affects animals of 6–24 months of age. Notable symptoms are bloody diarrhea, fever, anorexia, ataxia (lack of muscle coordination), and general weakness. While similar in clinical presentation to hemorrhagic syndrome it differs in that it only occurs in persistently infected animals, two biotypes of virus are present, and it is 100% fatal (Goyal and Ridpath, 2008) with death occurring within about 2 weeks after onset of clinical signs (Baker, 1995).

BVDV Immunosuppression

BVDVs are lymphotropic and acute infections cause reduction of circulating lymphocytes and suppression of innate immune functions. Decreased number of circulating lymphocytes may be the result of trafficking from blood into tissue, a reduction in leukogenesis, or outright cell death (Ridpath, 2010). Impact on the innate immune system in response to BVDV infection includes suppression of interferon production, phagocytosis, and microbicidal killing. It has also been reported BVDV causes immunosuppression of the adaptive

immune response with varying degrees of immunosuppression depending on the virus strain (Chase, 2013). Critical immunosuppressive mechanisms that impact that adaptive or acquired immunity are downregulation of major histocompatibility complex II and interleukin-2 that suppress T-helper cell response and apoptosis of T and B cells in lymphoid tissue.

Synergistic effects have been reported between BVDV and several viral and bacterial pathogens that are associated with Bovine Respiratory Disease (BRD) (Ridpath, 2010).

Synergism may occur by several different routes depending on coinfecting pathogens and target tissues. One possible result of synergy is increased dissemination of pathogens in tissues.

Experimental studies found that acute infections of BVDV enhanced susceptibility to infection of bovine herpes virus 1 and *Manheimia haemolytica* (Larson, 2015).

Persistent Infection

In addition to horizontal spread between animals, in pregnant animals, all pestiviruses can also be transmitted vertically by crossing the placenta and infecting the fetus of immunocompetent cows in the breeding herd (Meyers et al., 1996). Bovine viral diarrhea virus (BVDV) has the ability to cross the placenta and infect the fetus. Infection of a bovine fetus with a non-cytopathic strain, but not cytopathic strain, of BVDV in the first trimester of gestation can result in persistent infection (PI) of calf when born (Ridpath, 2010). Establishment of fetal persistent infection results in life-long viremia, virus-specific immunotolerance, and may have detrimental developmental consequences (Campbell, 2004). PI animals are a major source of virus among newly arrived feedlot cattle, and pose a significant threat for spreading the virus and establishing acute or primary infections in naive cattle. McClurkin et al. were able to persistently infect calves by exposing seronegative cows to non-cytopathic BVDV strains between 42 and 125 days of gestation. He followed the fate of the PI calves he generated and observed the

following: while many PI animals appeared weak and had congenital malformations, some appear apparently normal, while that majority of PI animals died soon after birth, some lived to breeding age, PI lines of cattle could be generated by breeding PI animals and PI animals spontaneously developed MD (McClurkin et al., 1984).

There has been variation in prevalence of PI cattle depending on the population sampled. The U.S. Department of Agriculture's National Animal Health Monitoring System NAHMS surveyed 205 beef operations in 24 States to determine prevalence of BVDV persistent infection in cow/calf herds. As part of this national survey, the beef operations submitted ear notches of their cattle to be tested for BVDV. The prevalence of BVDV among the tested ear notches was 0.12 percent (53/44,150). Within herds, the prevalence ranged from 0 to 16.0 percent. However, of the 205 operations that submitted ear notches, 18 had 1 or more positive samples, for a herd-level prevalence of 8.8 percent. Despite the low prevalence at the animal level, approximately 1 of 12 operations had at least 1 PI calf, suggesting that a number of operations likely have BVDV circulating in their herds (USDA APHIS, 2010).

The prevalence of BVDV PI cattle among all animals tested in 3 separate feedlot studies was between 0.3% and 0.4%, which included the some of the largest sample populations to date (Hessman, 2009), (Lonergan et al., 2005), (Fulton et al., 2006). A recent study concluded Southeast auction market stocker calves weighing <180kg were 2.78% more likely to be PI than calves > 180kg (Stephenson et al., 2017). Although total prevalence in these studies may seem low, population density in a feedlot environment also plays a major role in exposure outcomes. As the density of the population increases, exposure to PI cattle will increase as a result of an increase in frequency of contacts and, possibly, the duration of those contacts. This will increase the rate of exposure as well as the magnitude of the exposure (Hessman, 2009). In the feedlot

setting, production practices invariably result in new arrivals being placed in pens adjacent to cattle that have been at the feedlot for considerable time. Unlike some other food production systems in the United States, feedlots do not operate on an all-in all-out basis. Persistently infected cattle, therefore, have the potential to provide long term high-level exposure to pen mates and adjacent new arrivals, even if no PI animal is within a pen of new arrivals (Loneragan, 2005).

To control BVDV effectively, the cycle of PI calves needs to be broken as the PI animals are considered by most to be the major reservoir of infection (Fulton, 2013). Unfortunately, the identification of PI animals within a herd is confounded by the presence of colostral antibodies, thus, additional tests at a later date are required to confirm whether a young virus-positive animal is or is not PI with BVDV (Goens, 2002).

BVDV and Bovine Respiratory Disease

Bovine respiratory disease (BRD) is endemic and one of the most common and costly diseases in North America feedlots (Griffin et al, 1997). BRD is estimated to cost the cattle industry a total of \$500 million per year (Miles, 2009). Although not the primary site of replication, it has been shown that BVDV can establish infections in the respiratory tract of cattle. Infections can result in damage to the epithelial surfaces of the respiratory system and depletion of lymphoid tissue associated with the respiratory tract, but majority of these cases are subclinical (Ridpath, 2010). Loneragan and colleagues concluded persistently infected cattle are 43% more likely to require treatment for BRD and either become chronically ill or die than cattle that are not PI. In addition, they are associated with an increase in the incidence of BRD of in-contact cattle. 15.9% of initial treatments for respiratory tract disease among all cattle in the study were attributable to exposure to an animal PI with BVDV (Loneragan et al., 2005).

Although BVDV can cause clinical symptoms of BRD, numerous studies have concluded that BVDV association with BRDC is most importantly due to suppression of the immune system and synergism with other pathogens(Ridpath, 2010), (Peterhans et al., 2003), (Chase et al., 2004).

BVDV Reproductive Impact

Reproductive losses may be the most economically important consequence associated with BVDV infection and evidence suggests the incidence of BVDV-related reproductive losses are increasing in the United States (Evermann, 2002). Field and epidemiologic studies suggest that BVDV can have a significant impact on early reproductive performance. Exposure at time of breeding up to 45 days post service significantly reduces conception rates by disrupting normal fertilization and instigating early embryonic deaths and abortion (Grooms, 2004). Fetal infection between 100 and 150 days of gestation, often results in the development of a variety of congenital defects. In addition to reduced reproductive efficiency, BVDV uses the reproductive system to maintain and spread itself in the cattle population by inducing immunotolerance following fetal infection. Fetuses that survive infection with noncytopathic BVDV between 42 and 125 days of gestation, invariably develop immunotolerance to the virus and subsequently become persistently infected with BVDV, becoming a lifelong reservoir of virus (Mclurkin et al., 1984).

The introduction of BVDV to a naïve herd of breeding cattle can cause an outbreak with devastating effects on productivity. In one case, 136 females, confirmed bred, were purchased with no vaccination history; subsequent findings concluding a transient exposure BVDV2a. Of the 128 calves born (8 aborted), 8 died within 2 weeks after birth, 9 were born with congenital abnormalities such as corneal opacity, alopecia, and red hair, and 5 more died before 3 months of

age. Thirty-six were confirmed PI, and 19 more died of chronic illness or mucosal disease before reaching slaughter weight (Kane et al., 2011).

Economic Impact

The economic impact of BVDV on cow-calf operations is variable with key determinants being the timing of introduction of BVDV to a herd and management characteristics such as duration of the breeding season. Estimations of the annual cost of BVDV is roughly \$1.5- \$2.5 billion dollars (Ishmael, 2016). In a randomized controlled clinical trial, introduction of PI animals to seronegative heifers at 50 days prior to a controlled breeding season with constant exposure until mid-gestation was associated with no negative impact on health or reproduction (Rodning et al., 2012). In contrast, a later study indicated a negative health impact in 34% (46/136) of pregnancies associated with BVDV exposure of 3-year-old cows during gestation (Darweesh et al., 2015). In that report, eight cows exhibited early embryonic death or abortion, 8-week calves died during the first week of life, five PI calves died at weaning, and 25 PI calves died or were euthanized prior to 17 months of age. In a simulation model of production scenarios in USA beef cow-calf operations in 2002, there was an economic advantage for herds without PI calves of \$14.85 to 24.84 per cow (Larson et al., 2002). The economic damage accredited to BVDV exposure goes beyond the cow/calf sector. Hessman and colleagues reported that exposure of the general population of feedlot cattle to BVDV PI animals. Economic analysis of cattle with maintained exposure to a PI individual resulted in losses of \$88.26/animal and \$5.26/animal due to negative effects on performance and increased fatalities, respectively, totaling \$93.52 per head on 15,348 animals, for an overall loss over \$1.4 million (Hessman, 2009).

Vaccination and Control

Before the production of the first modified live vaccines in the 1960s, the spread of BVDV was limited to prevention of contact with infected animals (Ridpath, 2013). The goals of vaccination are to control the spread of an infection if the virus is introduced and to reduce the magnitude of clinical disease caused by the virus (Ridpath, 2013). That being said, the implementation of vaccines as a control strategy alone have not eliminated related clinical disease and losses attributed to BVDV (Lindberg et al., 2006). Control by vaccination can be compromised, not by lack of efficacy in available vaccine, but more so by the heterogeneity observed among BVDV strains, lack of complete fetal protection elicited by vaccination, and the failure to remove PI animals from cattle population (Ridpath, 2013).

Both killed and modified-live vaccines are available for the prevention of BVD. Modified-live vaccines contain an attenuated live antigen that replicates in the animal and more closely mimic a true infection response, whereas killed vaccines contain an inactivated or killed antigen that is incapable of replicating within the animal's body, thus requiring a higher initial antigen load in killed vaccines. In comparison to MLVs, killed vaccine have to be injected several times to achieve protection, and onset of immunity takes at least three to four weeks, whereas MLVs confer protection within a few days of vaccination (Huston, 2014). Many producers prefer to use MLV because the general consensus is that MLV offer broader protection with longer immune duration (Ridpath, 2013). However, the immune response to killed vaccines has been improved in recent years by adding powerful adjuvants. Humoral immunity after the application of killed vaccines is usually strong, and the cellular immunity varies from incomplete to strong (Moening 2018). In addition, the potential for immunosuppression by MLV vaccines exists (Fulton, 2015). MLV vaccines have the potential to cause a mild infection, and may not be

safe to use in all classes of animals such as pregnant or nursing cows. However, these cattle in fact can be safely vaccinated with MLV vaccine when label directions are strictly followed. Safety concerns have prompted the development of killed vaccines, which could be applied at any age and stage of pregnancy. Handling of and stability of MLV and Killed vaccines differ. In contrast to the killed products, MLV vaccines must be reconstituted and be used within a couple hours. They are also more sensitive to temperature and light variations. (Huston, 2014).

Most modern MLVs are cp BVDV, because cytopathic viruses are not able to establish a persistent infection in the fetus. In some cases, BVDV MLV vaccines have the potential to contribute to the development of post vaccination mucosal disease if a vaccine containing a CP strain is administered to a PI calf containing and antigenically similar NCP strain (Ridpath, 2013). Commercial vaccines contain BVDV1a and can also include BVDV2 antigens. Common BVDV1 and BVDV2 strains found in modified live vaccines are: Singer, NADL, C24, and GL 760 (NCP); 296, 5912, 53637, 125A, and NAH 1024 (NCP), respectively.

One goal of utilizing vaccination against BVDV is to prevent clinical disease following exposure to BVDV. Results of a 2016 study indicated that administration of any of 4 commercially available multivalent MLV vaccines that contained antigens against BHV1, BVDV1, BVDV2, PI3V, and BRSV to early-weaned beef calves with maternally derived antibodies prevented clinical disease, resulted in an increase in SNA titers against BVDV, and reduced the incidence of BVDV viremia and shedding (Walz 2016). Fulton suggested that MLV vaccines may induce higher antibody titers in calves than antibody titers from killed vaccines at time of vaccination (Fulton, 2000). Results from a recent study indicate MLV vaccines containing Singer strain induced higher virus neutralization levels to BVDV1a and BVDV1b than NADL vaccine in all three studies (Fulton, 2020). In the United States, it has been

demonstrated that titers 128 and higher provide protection from fetal challenge with BVDV1b (Leyh et al., 2011). Two vaccines, both MLV, containing Singer strain induced a higher proportion of 128 or higher BVDV1b titers than vaccine with NADL.

The other goal of vaccination is to prevent fetal infection that leads to the birth of persistently infected calves (Ridpath, 2013). Prevention of PI calves is important for control of BVDV in the cattle industry because PI cattle are believed to be the major source for viral exposure of susceptible cattle (Leyh et al, 2011). Two studies investigated the fetal protection of killed vaccines in breeding herds exposed to a PI animal. They reported that even with 2-4 doses of a killed vaccine pre-breeding, viremia and birth of PI calves were still observed (Walz, 2010; Grooms et al, 2004). Leyh and colleagues concluded that 1 dose of an MLV vaccine containing BVDV1a and BVDV2a at minimum immunizing doses in addition to other immunogens administered before breeding reduced the risk of fetal infections by 85% in pregnant heifers exposed to BVDV1b via PI cattle (Leyh et al., 2011). Although protection was not complete with only 1 dose of vaccine, evidence from this study indicated that the MLV BVDV1a and BVDV2a vaccine helps confer protection against a heterologous BVDV strain, BVDV1b (Leyh et al., 2011). Rodning et al. indicated commercial vaccines provided effective fetal protection despite prolonged natural exposure to BVDV. That being said, viremias were detected in 11 vaccinated heifers after BVDV exposure, and two vaccinated heifers gave birth to persistently infected calves. Close attention to biosecurity and diagnostic surveillance, in addition to vaccination, is crucial to ensure effective BVDV control (Rodning et al. 2010).

Prevalence of Subspecies

As heterogeneity of BVDV allows for antigenic differences among subspecies, several studies have focused on the predominance of the three BVDV subspecies known to circulate United States cattle herds. Early studies showed BVDV1a to be the most prevalent (Ridpath, 1994). Follow up studies by Ridpath et al. indicated the decrease of BVDV1a over a 20-year span in accessions submitted solely from Texas (Ridpath et al, 2011). Though the three sequential studies indicated a higher prevalence of BVDV1b accessions between 1988 and 2008, results could not be extrapolated to a nationwide predominance, as isolates were sourced from a highly conserved geographic region. However, multiple surveys from various segments of the cattle industry have also reported a common trend of predominating BVDV1b isolates.

Diagnostic laboratory accessions from 26 dairy operations in the United States by use of bulk milk samples and samples from infected dairy cattle indicated that the prevalence of BVDV1b, 1a, and 2a were 49.1%, 11.3%, and 39.33%, respectively, from 53 isolates (Tajima, 2005). A diagnostic laboratory study using BVDV positive isolates from Oklahoma, Texas, Arkansas, and Kansas reported that 45.8% were BVDV1b, 28.2% were BVDV1a, and 26.0% were BVDV2a (Fulton, 2005). As BVDV has been associated with BRD and other diseases in feedlots (Larson, 2015), feedlot prevalence has also been investigated. A feedlot study evaluating diagnostic methods of BVDV detection from Southern and Southeastern, order-bought, 86 PI cattle were identified. Distribution of BVDV subtypes was BVDV1b (77.9%), BVDV1a (11.6%), and BVDV2a (10.5%) (Fulton, 2006).

Diagnostic Methods

Advances in BVDV diagnostic science have led to several methods of BVDV identification. Traditionally, virus isolation was the most frequently used technique (Haines et al,

1992) and still remains the gold standard. In the live animal, the best sample for BVDV isolation is whole blood from which white blood (buffy coat) cells are extracted and used as the inoculum. The best necropsy or aborted fetus samples are lymphoid organs such as spleen, Peyer's patches from the small intestine, mesenteric lymph nodes, and thymus. In PI animals, the amount of virus present in the organism is so high that virtually any secretion, excretion or tissue sample will be satisfactory for BVDV isolation. Incubation of 4-5 days is sufficient for virus isolation (Saliki, 2004). Since NCP strains do not cause visual cytopathic effect, further testing using fluorescent monoclonal/polyclonal antibodies are needed to confirm presence of NCP BVDV strains. For handling of large numbers of samples such as in whole herd screening for PI cattle, a microtiter virus isolation method, the immunoperoxidase monolayer assay (IPMA), using serum as the diagnostic specimen is widely used (Saliki et al., 1997).

Immunohistochemical (IHC) staining for diagnosis of persistent BVDV is performed on skin (ear notch) samples fixed in 10% formalin by observing sample tissue interaction with anti-BVDV monoclonal antibodies (Fulton, 2006). A strength of this testing method is the use of modified-live BVDV vaccines does not result in false-positive results (Dubois, 2000). Positive IHC results are characterized by distinct red granular intracytoplasmic staining in the epithelium of the stratum spinosum and stratum basale of the epidermis and follicular infundibulum in more than one location (Fulton, 2009).

Serology tests measure antibody response of animals exposed to BVDV through natural exposure or vaccination protocol. Common forms of serology are ELISA and serum/virus neutralization with ELISA being less preferred due to extensive viral diversity observed among BVDV isolates (Saliki, 2004). Serum neutralization, which provides a titer of antibody, is subject to variation by the strain of BVDV that is used and the test cells. Since not all diagnostic

laboratories use the same BVDV strain and/or test cells, there is possibility that varying results will be observed between testing centers (Carman et al., 1998). When applied correctly, serology tests have the capability to assess vaccine efficacy, assess vaccination protocol compliance, assess herd status as to exposure to BVDV, and associate BVDV with clinical signs (Saliki, 2004).

PCR stands for polymerase chain reaction and is another commonly used BVD diagnostic tool. PCR amplification of an RNA genome involves the binding of specific DNA oligonucleotides to cDNA target sequences, resulting in amplification of size-specific DNA fragments that are detectable by gel electrophoresis (Gilbert, 1999). The high analytical sensitivity of RT-PCR allows for pooling of specimens to reduce unit test cost (Kennedy 2006). Pooling is especially applicable for persistent infection testing whereby a single positive specimen can still be detected in a pool of several dozen samples. Polymerase chain reaction (PCR) using primers specific for the 5' untranslated region (UTR) of the Bovine viral diarrhea virus (BVDV) genome is performed to amplify a quality product for subsequent DNA sequence analysis (Ridpath, 1994). DNA sequencing of the PCR product is done to determine similarities or differences in the nucleic acid composition of the sample as compared to known field isolates. Sequences are used as an estimation of relatedness and are not a direct measure of virulence. Identification of BVDV RNA by 5'UTR PCR, is determined positive based on the detection of a PCR product that migrates to the approximate fragment size position as the BVDV positive control (Brock et al., 1992). Of the three commonly used BVDV detection methods, real-time PCR is the only test with 100% expected sensitivity and specificity.

Objectives

Previous research has revealed a predominance in BVDV1b as the most prevalent subspecies isolated. However, the indication of national prevalence is confounded by the highly conserved geographic regions in prior studies. Essentially, there is a lack of nationwide data regarding BVDV subspecies prevalence from all aspects of cattle production. That is why the overall objective of this research is to determine the prevalence of BVDV subspecies 1a, 1b, and 2a in cattle previously identified as PI from various sectors of the cattle industry, sourced from different geographic regions across the United States. Comparing the prevalence and distribution of Bovine viral diarrhea virus in different sectors of the U.S cattle industry to previous prevalence studies may aid in identifying changes needed at different stages in the production cycle for more effective control measures.

Chapter 2 - Prevalence of Bovine Viral Diarrhea virus Subspecies Among Persistently Infected Positive Samples Submitted to a Diagnostic Laboratory From Cattle in the United States

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is an important infectious disease affecting cattle worldwide. The term Bovine viral diarrhea virus refers to a heterogeneous group of single stranded RNA viruses, within the Pestivirus genus of the Flaviviridae family. Viruses can exist as one of two biotypes, cytopathic, or non-cytopathic based on the cytopathic effect when observing infected cell cultures (Baker, 1995). Non-cytopathic strains predominating in nature however, most commercially available vaccines contain a CP strain of BVDV (Baker,1995).

BVDV is complex disease syndrome with varying virulence and clinical presentation, that range from unapparent infections to severe fatal, systemic diseases, such as mucosal disease(MD) (Goyal and Ridpath, 2008). The greatest impact of BVDV is its association with respiratory and reproductive disease. Its impact on the bovine respiratory disease (BRD) is the result of immune dysfunction that leads to opportunistic infections with other pathogens. Its main reproductive impact results in persistent infection. Depending on age of fetal exposure, BVDV infection can result in abortion, stillbirth, congenital defects and persistent infection (Grooms, 2006). Successful infection of a fetus with a non-cytopathic strain of the virus approximately 42-125 days of gestation can result in the birth of a persistently infected calf (McClurkin et al., 1984). PI calves are immunotolerant to the virus that caused fetal infection and serve as lifelong reservoirs, shedding copious amounts of virus through all bodily secretions and excretions (Givens, 2015).

Phylogenetic analysis utilizing polymerase chain reaction and nucleotide sequencing has differentiated BVDV into two species BVDV1 and BVDV2 (Ridpath, 1994). Further investigation identified 3-21 subspecies within each species (BVDV1a-u, BVDV2a-c) (Yesilbag, 2017). In the United States, there are three main subspecies circulating cattle herds: BVDV1a, BVDV1b, and BVDV2a. One study reported a strong decline in BVDV1a samples submitted to the same diagnostic laboratory over a 20 year span (Ridpath, 2011). Subsequent studies have identified BVDV1b as the predominant strain among PI cattle and in BRD (Fulton, 2002), (Ridpath, 2010), (Loneragan, 2005). The prevalence of each genotype is important as it relates to vaccine use for control and diagnostic testing. Vaccines commercially available in North America typically contain a BVDV1a strain and often a BVDV2a strain. However none of the major vaccines have a BVDV1b strain (Fulton 2015). Although some studies (Fulton, 2020) (Leyh, 2011), (Schnackel, 2007) have observed cross protection, vaccines with BVDV1a and 2a components may not provide adequate protection against BVDV1b. The purpose of this surveillance study was to determine the prevalence of BVDV subtypes 1a, 1b, and 2a in cattle previously identified as PI from various sectors of the cattle industry, sourced from different geographic regions across the United States.

MATERIALS AND METHODS

In the fall of 2018, the professional service veterinarians and territory sales representatives from an animal health company^a began the nationwide initiative to gain further insight on the prevalence of BVDV subspecies circulating throughout the United States. Cattle producers, veterinarians, and regional testing centers were individually contacted to determine their interest in determining the species and subspecies of BVDV in confirmed positive PI cattle. Sample submission was completely voluntary. Committed participants provided ear notch

samples from stored (in a freezer) previously confirmed PI samples and future PI positive samples during the study timeframe. Various diagnostic methods were reported by private testing centers analyzing ear notch samples to determine PI status including: polymerase chain reaction (PCR), antigen capture- ELISA, and immunohistochemistry (IHC). There was not preference or bias of previous testing methods from the primary investigators.

Ear notch samples from confirmed or suspected PI animals were submitted by veterinary clinics and producers between November 1, 2018 through December 20, 2019. Animal health company representatives managed submissions and would utilize an “All Species Herd Health Form” with owner information, number of samples included, and the test to be ran, BVDV typing PCR. If a veterinarian had frozen samples stored for multiple producers, accessions for each producer would be separated into gallon zip-loc bags with a submission included in both and shipped overnight on cool packs. However, some testing centers submitted multiple samples under one entity, to preserve individual producer anonymity. Over the approximately 1-year span 1,093 samples were submitted to the Animal Disease Research and Diagnostic Laboratory(ADRDL) at South Dakota State University. Once received by ADRDL, samples were then verified positive or negative for the presence of BVDV by polymerase chain reaction (PCR) of the 5’ untranslated region (UTR) of the viral genome as described in (MOL.SOP.0041.2). If identified as positive, PCR products of 5’ UTR were sequenced and typed as BVDV1a, BVDV1b, or BVDV2 based on nucleotide percent homology to sequences stored in BLAST database..

RESULTS

One thousand and ninety-three samples (1,093) samples were submitted for processing to the Animal Disease Research and Diagnostics Laboratory of South Dakota State University. The

initial screening PCR used by ADRDL detected 1,000 samples as BVDV PI positive. Positive submissions were supplied from 21 states with sample size ranging 1-288 samples/state (Table1). Samples from 4 states: Kansas(n=258), Kentucky(n=288), Oklahoma(n=237), and Texas(n=81) accounted for 86.4% of total accessions. Samples were predominately obtained from beef operations, with 12, 3, 1, and 1 samples sourced from dairies in California, New York, Connecticut and Wisconsin, respectively. Of the PCR confirmed samples, the prevalence of subspecies was BVDV1b 702/1,000 (70.2%), BVDV1a 44/1000 (4.4%), and BVDV2a 178/1000 (17.8%), respectively, with 76/1000 (7.6%) of samples unable to be translated successfully. The 76 samples unavailable for subtyping was attributed to either low concentration of BVD nucleic acid or noisy multiple peak sequence data, preventing sequencing of 5' UTR.

CONCLUSION

A pool of 1,000 samples were diagnosed positive for persistent infection of bovine viral diarrhea virus via PCR. Results of sequencing the 5' UTR of the viral genome showed that BVDV1b was 3.5 X (70%) more prevalent than BVDV2a (17.8%), and BVDV1a was the least at 4.4%. These findings support previous research suggesting BVDV1b is the predominant strain circulating the United States.

DISCUSSION

The focus of this survey was to become more educated on the national prevalence of BVDV subspecies in the United States without limits to a specific geographic region. In the current study, a distinguishable difference was observed in the prevalence of BVDV subspecies isolates submitted. Following PCR sequencing and comparison of the 5' UTR, all isolates were differentiated into one of two species: BVDV1 or BVDV2, then 1 of 3 subspecies: BVDV1a,

BVDV1b, BVDV2a. The predominant species among samples was BVDV1, with the predominant subspecies overall being BVDV1b (>70%), found in Table 2.1.

Analysis of BVDV samples during 2018 and 2019 offered insight as to what BVDV types are circulating in the cattle population, encompassing the most states (n=21), with a larger set of isolates compared to earlier studies(Ridpath 2011, Fulton, 2006). The results of this study indicated a higher prevalence of BVDV1b than a previous study(Fulton, 2005) and lower than two more recent studies that reported prevalence >75% (Fulton 2006, Ridpath, 2010), with each study exhibiting BVDV1b predominance over BVDV1a and BVDV2.

The distribution of subspecies varied by state (Table 2.2). A total of 22 samples from 8 states(AL, CA, CT, GA, IA, MS, NC, NY) had only BVDV1b isolates. Not a single state exhibited a predominance of BVDV1a, with several states having zero BVDV1a accessions. A large sample set from the state of Kansas (n=258) represented cow/calf, stocker, and feedlots. All of the samples submitted from feedlots in the study were sourced from Kansas. Nearly 82% of Kansas samples were identified BVDV1b. With that in mind, cattle in commercial feedlots can be sourced from different states of various geographic regions, so this could be even more indicative of widespread BVDV1b predominance.

BVDV was segregated into two species, BVDV1 and BVDV2 in the 1990's after fetal infection was found in heifers vaccinated for BVDV1a. There was a BVDV2b, later designated to BVDV2c, isolate identified in a fatal feedlot pneumonia case (Fulton et al., 2009). However, the virus never surfaced as an impactful subspecies, despite the lack of a 2b or 2c strain in any vaccine. More diversity in BVDV subspecies has been documented globally. Epidemiological studies have shown that various BVDV subspecies predominate in different countries (Yesilbag, 2017). Phylogenetic analysis showed BVDV1a and BVDV1c subspecies currently circulate

eastern China where no vaccination had been used. Of the 36 Chinese dairy herds included in the study, 77.8% tested positive for BVDV antibodies with a PI prevalence of 1.86% (Hou 2019). In South Africa BVDV1a-d is found throughout the region, with no single subspecies exhibiting predominance over others. This lack of predominance might be attributed to less immunological pressure by extensive vaccine use.

If vaccination is utilized in Europe, mainly killed vaccines are utilized for many different reasons such as legal availability (Moening, 2018). The Scandinavian countries and Austria do not permit the use of BVDV vaccines (Lindberg, 2006). Instead, large-scale eradication programs are in place in several European countries (Stahl, 2012). Eradication programs in Scandinavian countries began in the 1990's to control BVDV through the identification herd BVDV status, systematic biosecurity, and to reduce the prevalence of infected herds by identification and elimination of PI animals. Scandinavian countries are currently either free, or almost free from BVDV. All cattle were directly tested for BVDV in the year 2008 and all newborn calves until the end of 2012, where the PI prevalence had dropped to 0.02%. Vaccination remains prohibited (Bachofen, 2013). Due to the drastic differences between the cattle industries of Scandinavian countries and the US, similar undertakings have not been attempted in the United States. Sweden had <475,000 calves born in 2018 (SBC, 2019). In a summer census of the Danish cattle population, approximately 527,000 calves <1 year-old were identified. A recent report from the USDA showed a 2019 calf crop in the United States was estimated at 36.1 million head (NASS, 2019). A limitation in the application of a similar BVDV eradication initiative in the United States is vast a difference in cattle population.

Vaccines against bovine viral diarrhea viruses (BVDV) have been available in the U.S. since the 1960's and prove to be efficacious under controlled conditions. However, the

utilization of vaccination alone has yet to eliminate BVDV as a source of significant losses for producers (Ridpath, 2012). Although effective vaccines are widely available, there are inconsistencies in BVDV vaccination as an industry. In a cow/calf survey (USDA APHIS, 2010) only 4.2% of operations had tested any calves for persistent infection with BVDV in the previous 3 years. Overall, 46.6 percent of producers were unsure if removing calves that tested positive for persistent infection with BVDV would affect the value of the remaining calf crop. The survey revealed 33.1% of operations vaccinated calves against BVDV at 22 days of age through weaning, 25.1% vaccinated weaned replacements heifers through breeding, and only 28.1% vaccinating cows prior to breeding.

Preventing fetal infection is the utmost priority for reducing the incidence of BVDV in herds as the birth of live PI calves serve as lifelong reservoirs for virus shedding. Past vaccination studies have presented varying results on vaccination efficacy in preventing fetal infection in immunocompetent breeding females. A Brazilian dairy herd still experienced low conception rates and identified PI cattle despite biannual vaccination containing a killed NADL strain of BVDV1a (Otonel, 2013). Interestingly, one calf from a PI negative dam, died from BVDV1a infection, while the 2 PI cows in the herd were infected with BVDV1b and BVDV1d strains. Conversely, a meta-analysis by Newcomer et al revealed vaccination reduced abortions and fetal infections by 45% and 85%, respectively (Newcomer et al., 2015). In the United States, titers of 128 and higher provide protection from fetal challenge with BVDV1b (Leyh, 2011). A virus neutralization titer of 128 and higher was previously found to provide protection in heifers against BVDV1b challenge with the Singer strain induce significant level of BVDV1b antibodies, thus use of the vaccines with Singer strain potentially provide greater protection

against the BVDV1b strain (Fulton 2020), the most common BVDV strain in the U.S. cattle population.

Further studies are necessary to develop a clearer image of the distribution of BVDV subspecies. The overall scope, various segments, and population density of fed-cattle operations present complex challenges to systematic control in United States cattle. Follow-up studies should include accurate history on calf origin of isolates as this could give more accurate insight on true incidence of BVDV, and prevalence of subspecies, based on region. More research on the distribution of BVDV subspecies by geographic region is essential. Although the study reported here encompassed several states, a significant portion of isolates came from states that have been studied previously. Having a representative number of samples from all regions of the United States would be warranted to further understand the true prevalence of the subspecies of PI animals being produced, and their potential geographic differences. Furthermore, antigenic variation between BVDV subspecies suggests vaccine protection may be improved by utilizing vaccines that best demonstrate cross-protection efficacy of towards BVDV subspecies of importance with respect to geographic regionality. Overall, more consistent, judicious use of vaccines, removal of persistently infected animals, and prevention of incorporating untested cattle into the breeding herd should be implemented to effectively mitigate BVDV.

Endnote

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Table 2.1 Overall BVDV subspecies prevalence samples from 1,000 positive persistently infected cattle

	Positive Samples			
	BVDV1a	BVDV1b	BVDV2	UT
Total No. Positive	44	702	178	76
Prevalence (%)	4.4%	70.2%	17.8%	7.6%

Table 2.2 Total sample submission and prevalence of BVDV subspecies per state

State	# of Pos. Samples	BVDV Subspecies			
		1a	1b	2	UT
Alabama	1	0	1 (100)	0	0
Arkansas	28	5 (17.9)	7 (25)	13 (46.4)	3 (10.7)
California	12	0	12 (100)	0	0
Connecticut	1	0	1 (100)	0	0
Florida	14	1 (7.1)	12 (85.7)	1 (7.1)	0
Georgia	1	0	1 (100)	0	0
Iowa	2	0	2 (100)	0	0
Idaho	8	0	7 (87.50)	1 (12.1)	0
Indiana	10	1 (10)	8 (80)	1 (10)	0
Kansas	258	12 (4.7)	211 (81.8)	25 (9.7)	10 (3.9)
Kentucky	288	11 (3.8)	177 (61.5)	83 (28.8)	17 (5.9)
Missouri	26	1 (3.8)	22 (84.6)	2 (7.7)	1 (3.8)
Mississippi	1	0	1 (100)	0	0
North Carolina	1	0	1 (100)	0	0
New York	3	0	3 (100)	0	0
Oklahoma	237	8 (3.4)	170 (71.7)	21 (8.9)	38 (16)
Tennessee	7	0	4 (57.1)	3 (42.9)	0
Texas	81	5 (6.2)	54 (66.7)	19 (23.5)	2 (2.5)
Virginia	12	0	4 (33.3)	6 (50)	2 (16.7)
Wisconsin	1	0	0	0	1 (100)
West Virginia	8	0	4 (50)	1 (12.5)	3 (37.5)

*Numbers in parenthesis are percentages

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