PREVALENCE OF STOCKER CALVES PERSISTENTLY INFECTED WITH BOVINE VIRAL DIARRHEA VIRUS IN THE SOUTHEAST DETERMINED USING IMMUNOHISTOCHEMISTRY ON SKIN BIOPSIES

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A Thesis

Submitted to

the Graduate Faculty of

Auburn University

in Partial Fulfillment of the

Requirements for the

Degree of

Master of Science

Auburn, Alabama
May 9, 2009

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THESIS ABSTRACT

PREVALENCE OF PERSISTENTLY INFECTED STOCKER CALVES IN THE SOUTHEAST DETERMINED USING IMMUNOHISTOCHEMISTRY ON SKIN BIOPSIES

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Master of Science, May 9, 2009
(DVM, Auburn University, 2008
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55 Typed pages

Directed by Kenny V. Brock

Bovine viral diarrhea virus (BVDV) is an insidious disease affecting cattle worldwide. It can cause early embryonic death, congenital defects, abortions, respiratory disease, immunosuppression, reproductive failure and may cause mortality. Animals that are born persistently infected (PI) with BVDV are considered the direct viral reservoirs that shed copious amounts of virus into the environment through aerosols, mucus secretions and fecal matter. Stocker calves from auction markets in the Southeastern USA (Alabama, Florida, Georgia, Mississippi and Tennessee) were sampled

from March to December 2005. Once organized into average weight groups, the calves were processed and skin biopsies were collected in zinc buffered formalin for the detection of BVDV persistent infection using immunohistochemistry. Twenty four BVDV positive calves were detected of the 7,544 calves sampled. Confirmation testing at a later date was not an option in this study. The overall BVDV-PI prevalence rate of stocker calves sampled in the Southeast was estimated at 0.3%. This prevalence rate compared closely, if not less than, other PI prevalence rates reported in other areas of the U.S. In addition, calves in the less than 400 lbs. weight group had a 2.78 times higher probability of having a PI animal present when compared to the greater than or equal to 400 lbs. weight group. Therefore, a PI prevalence rate of 0.3% was found in stocker calves sampled from the Southeastern U.S. and the low weight groups less than 400 lbs. had a 2.78 times greater chance of having a PI animal in that group when compared to the heavier weight groups.

Keywords: Bovine viral diarrhea virus, persistent infection, prevalence rate

ACKNOWLEDGEMENTS

The author would like to thank her mentor, Dr. Kenny Brock, for his guidance, support and patience throughout the course of this research project and veterinary school. The author would also like to thank Terri Hathcock, Terri Wood, Shailah Armstrong and Elizabeth Landreth for technical support, as well as Roberto A. Palomares for statistical support. The author offers further gratitude to Dr. Brad White and Dr.Terry Engelken for providing samples for this project and Dr. Dan Givens and Dr. Stephen Lenz for serving on this graduate committee. The author would also like to thank Philip Stephenson, Joe and Catherine Kassler for their love and support during this project.

Journal used: Journal of Veterinary Diagnostic Investigation

Computer software used: Microsoft Word

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CHAPTER I
INTRODUCTION

Bovine viral diarrhea virus (BVDV) is an insidious disease affecting cattle worldwide (25). The consequences of BVDV infection are significant for the naïve animal as well as vaccinated animals. Bovine viral diarrhea virus causes early embryonic death, congenital defects, abortions, respiratory disease, immunosuppression, and mortality (1). The development of BVDV persistent infections during fetal development *in utero* is unique to the pathogenesis of BVDV. Persistently infected (PI) animals occur following an acute infection of the dam prior to 150 days of gestation with a noncytopathic strain of BVDV (9). Following the acute replication of the virus in the susceptible dam during the first trimester, BVDV passes through an unknown transplacental pathway to infect and replicate within the immunologically incompetent fetus (1, 9). The newborn PI calf is immunotolerant to BVDV, without the ability to develop an immune response against antigenically similar strains of BVDV (14). Animals that are born PI are considered the primary viral reservoirs that directly shed copious amounts of virus into the environment through respiratory aerosols, mucus secretions and fecal matter (1).

As the primary reservoir of BVDV, these proliferative BVDV shedders are the key in the amplification of BVDV in the cattle population by direct and environmental

exposure to BVDV. Wittum *et al*. conducted a five state survey in beef cattle in the United States and reported finding a BVDV prevalence rate of 3% in a random selection of beef herds (68). Lonergan *et al.* reported a 0.3% PI prevalence of cattle entering feedlots from Kansas and Texas (43). While Fulton *et al.* found a 0.6% prevalence of PI animals from Texas and Oklahoma (23). In a study by Cornish *et al.* on Wyoming beef calves, 10.5% were found to be persistently infected after repeated testing (16).

Currently, significant measures are being taken to control and eventually eradicate BVDV from beef and dairy herds in the U.S. The focus of BVDV control programs is to first identify and then remove PI animals. Removal of a single PI animal eliminates a potent source of virus within the herd and reduces the number of cattle naturally exposed to the virus (24, 42). Another key component to control is the institution of biosecurity measures. In maintaining a biosecurity program, producers are encouraged to isolate new additions, avoid pregnant additions and confirm a BVDV negative status before introduction to the native herd. This is a preventive measure to negate the possibility of reintroduction of BVDV into the herd (42). Vaccination strategies are also important to reduce the impact of transient and fetal infections.

Successful BVDV control programs use diagnostic testing to first identify PI animals for immediate isolation, if retesting is an option, or removal before significant viral spread leads to herd morbidity, mortality or vertical transmission to early gestation dams (24). Several diagnostic techniques are available to detect persistent infection and allow veterinarians to accomplish producer's herd management goals. These options

include virus isolation, immunohistochemistry, reverse-transcriptase PCR, and antigen-capture ELISA.

The main objective of this study was to determine the prevalence of BVDV among stocker calves in the Southeastern United States. According to the literature, the majority of PI animals are defined as "poor doers" who do not thrive well in the production setting (1). The virus does not allow the PI animal to make significant gains in performance and they rarely survive to breeding age, however, exceptions do exist (1). Although it has been suggested that a nationwide prevalence of PI animals is approximately 3%, some have characterized stocker calves from the Southeast region as having increased BVDV disease rates, therefore it was important to determine if the prevalence of BVDV PI animals was comparable to infection rates in other areas of the United States (68). In this study, immunohistochemistry diagnostic testing on zinc formalin fixed ear notch tissue was used to detect BVDV antigen for determination of PI status. In addition, the study was designed to investigate whether cattle weight, more importantly if low weight cattle, served as a risk factor for acquisition of PI stocker cattle. If PI cattle are not as thrifty, it would seem reasonable to assume that a random group of low weight stocker calves would have a higher incidence of persistent infection than a random group of heavy weight stocker calves. Previous studies have focused on the low weight cattle group but did not include mid-weight or heavier cattle weights for comparison (53). Therefore, another goal of this study was to determine if purchasing groups of low weight cattle posed a higher probability of purchasing a PI stocker calf.

CHAPTER II

LITERATURE REVIEW

Bovine viral diarrhea virus was first reported in a New York state dairy herd in 1946 by Olafson, McCallum and Fox (52). They described clinically depressed, anorectic animals with leukopenia, diarrhea, mucosal ulcers in the nasal and oral passages, abortions and a high morbidity to mortality ratio (52). During the same year, Canadian researchers described a similar bovine disease but with more severe clinical signs of "fever, anorexia, depression, profuse salivation, nasal discharge, gastrointestinal hemorrhages, erosions, ulcers and severe diarrhea with watery feces, at times mixed with blood, followed by death" (15, 24). The differences in the severity of clinical signs led researchers to believe the two syndromes were not related but did share some of the same characteristics (24). Some time would pass before this more severe disease seen in Canada was found to be, in fact, another manifestation of BVDV, known as mucosal disease (MD) (24).

In the late 1950s, the two biotypes of BVDV, cytopathic and noncytopathic, were discovered (24). The cytopathic biotype produced lysis of infected cells in cell culture, while the noncytopathic biotype produced no appreciable change to infected cells. With the knowledge of the cytopathic biotype came the development of serum and plaque neutralization assays that used antisera to neutralize the BVDV antigen and to diagnosis

BVDV (24). Because of this new diagnostic tool, cases of cytopathic BVDV and mucosal disease from North America and Europe were studied and found to be related antigenically (24). It wasn't until the early 1960s, when further research was conducted on the cytopathic MD strains, using virus neutralization, that it was determined mucosal disease was in fact a syndrome of BVDV (24).

In 1963, Pritchard classified clinical BVDV disease into three forms: severe acute, mild acute and chronic BVDV (24, 59). The clinical signs ranged from a "harsh, dry cough" with or without laminitis, seen in the severe acute form, to "failure to grow at a normal rate or weight loss leading to emaciation with the development of continuous or intermittent diarrhea," seen in the chronic form of BVDV (24, 59). It was also found that the noncytopathic biotype responsible for the acute form of BVDV did not cause cytopathological changes in cell culture, making the study of acute BVDV difficult (24). In addition, Pritchard found that the mucosal syndrome of BVDV could not be reproduced in an experimental setting (24, 59). It was hypothesized that the immune system played a role in the pathogenesis of mucosal disease because animals were serologically negative to the virus, but viremic throughout the course of disease (24).

In the mid to late 1960s, modified-live vaccines containing the cytopathic virus were mass produced and considered effective against the acute form of BVDV (24). Consequently, several cases were reported of a few animals developing signs of mucosal disease in herds shortly after vaccination with the modified live cytopathic vaccine (24). As a result, a post vaccinal mucosal disease case was investigated and it was discovered that those animals that died after vaccination did not develop antibodies to BVDV. At the time, it was believed these cattle had failure of the immune system and therefore were

extremely susceptible to the disease, although the number of affected animals were few (24). Later, researchers realized that post vaccinal mucosal disease only occurred in animals persistently infected (PI) with BVDV (24). Persistent infection was identified when newborn calves were born with BVDV infections, which indicated an intrauterine route of infection, and were unable to clear the virus (24). Persistent BVDV infection of the fetus occurred before the fetal immune system was competent, leading to immunotolerance to BVDV and recognition of the virus as "self" instead of a foreign antigen. In these animals, anti-BVDV antibodies were not produced (24).

Originally, PI animals were perceived to be ill, poor doers that died in the first few months of life. In the 1970s, BVDV virus was collected from the semen of a healthy, seronegative, PI bull (1, 24). In 1979, McClurkin *et al*. conducted reproductive studies using a PI bull, three PI cows, and several BVDV seropositive, immunocompetent cows (24, 45). When the PI bull was bred to PI cows, they produced PI calves infected with the same noncytopathic virus as the dam and did not live beyond a few weeks of age (24, 45). However, the offspring of the PI bull-BVDV seropositive cows were healthy and free of BVDV (24, 45). In the same study, a PI bull was bred to five BVDV seronegative cows. The cows seroconverted, producing high antibody titers to BVDV. Four of the five cows produced normal calves. The fifth cow aborted at six months gestation, and BVDV was not isolated from fetal tissues (24, 45).

In addition, McClurkin noted a high service per conception rate when the PI bull was breeding the seronegative cows (45). BVDV can affect semen quality by causing abnormal morphology and a decreased sperm motility (1). Seronegative cows appear to experience fertilization failure when inseminated with BVDV infected semen until an

immune response is mounted against the virus (1, 45, 62). The veneral route of transmission of BVDV by natural service yields the highest risk of compromising fertilization in cattle leading to increased reproductive losses (1).

BVDV research increased dramatically in the 1980s. McClurkin *et al*. conducted studies using thirty-eight pregnant cows that were challenged with five different BVDV strains, four noncytopathic and one cytopathic, at 42-125 days gestation (24, 46). The results using the noncytopathic strain were "ten abortions, one stillborn calf, four weak or unsteady calves, and twenty-three calves that had a normal and vigorous appearance at birth (24, 46)." It was found that all four of the weak calves and twenty-two of the twenty-three normal calves were persistently infected with BVDV (24, 46). The lone calf that was not PI and immunocompetent against BVDV was inoculated at day 125 of gestation (24, 46). By following the PI calves after this study, researchers realized that PI animals can appear normal and live to breeding age producing more PI offspring and acute infections (24, 46). However, more importantly was the inability of researchers to produce a PI using the cytopathic strain in this study. Brownlie *et al.* also attempted to produce PI calves experimentally using a cytopathic strain of BVDV and were also unsuccessful (12, 24). As a result, it was determined that PI infections resulted from fetal infection with a noncytopathic strain of BVDV (12, 24).

In addition, mucosal disease was experimentally reproduced in healthy PI cattle (4, 11, 24). It was observed, that although the clinically healthy PI calves were inoculated with only noncytopathic BVDV strains, both noncytopathic and cytopathic BVDV strains were detected in clinically ill PI cattle with mucosal disease (11, 24). Subsequently, researchers demonstrated that mucosal disease resulted when PI animals

were ‘super-infected’, post-natally, with a cytopathic BVDV strain (4, 11, 24). Further analysis of the noncytopathic:cytopathic virus pairs from cases of MD determined that each biotype had to be antigenically similar to produce MD (4, 24). Thus, mucosal disease was re-defined to indicate that “mucosal disease was induced in a persistently infected animal by “superinfection” with a cytopathic BVDV with antigenic similarity to the noncytopathic BVDV (4, 24).” It was further determined that superinfection could occur by spontaneous mutation of the persisting noncytopathic strain or through vaccination with a virus that was antigenically similar to the noncytopathic virus causing PI (4, 24).

In the 1980s, BVDV was proven to play a role in the bovine respiratory condition known as “shipping fever” pneumonia. Shipping fever was the cause of substantial mortality in feedlots and thought to be due to the stress of transporting cattle across the nation (24). The pathogenesis of shipping fever was hypothesized to include a combination of a “pneumotropic virus and a colonizing bacterial species (24, 69).” From earlier studies it was evident that BVDV could induce mild respiratory disease, but studies using BVDV with and without the bacterium *Mannheimia haemolytica* proved BVDV could cause significant damage to the bovine respiratory system (19, 24). Potgieter *et al.* performed experiments to induce shipping fever using BVDV without a bacterium, *M. hemolytica* alone and BVDV with subsequent infection with *M. hemolytica* (24, 58). The group inoculated with BVDV only developed “mild respiratory tract lesions characterized by small, scattered areas of interstitial pneumonia involving 2-7% of the total lung volume (24, 58).” The group infected with the *M. haemolytica* bacteria alone had “localized lesions involving about 15% of the lung” and the group infected

with both BVDV and *M. haemolytica* "produced a severe fibrinopurulent bronchopneumonia and pleuritis involving 40-75% of the lung volume (24, 58)." It was apparent that synergism between BVDV and *M. haemolytica* bacteria proved a damaging combination for the bovine respiratory system and the feedlot industry.

Throughout the 1980's, research focused on the persistently infected animal. A PI animal was experimentally produced by vaccinating a pregnant dam with a modified live vaccine prior to approximately 125 days of gestation (24, 46). After this point in gestation, the fetus was immunocompetent and capable of mounting an immune response to the virus (24, 40, 46). Liess *et al.* were able to generate congenital malformations, such as cerebellar hypoplasia and hydrocephalus, in their studies of early gestational vaccine-induced BVDV increasing the awareness of PI production through use of modified live vaccines in breeding animals (24, 40).

In the 1970s and 1980's, several advancements were made in diagnostic testing to detect BVDV infections. In 1974, Lambert recommended using serology to diagnose BVDV infection with a serum neutralization assay on paired samples, collected 2-3 weeks apart (24, 38). Active BVDV infection was confirmed if a rising titer was present between the paired sera samples (24, 38). Lambert recommended vaccinating open heifers and cows 1-2 months prior to breeding for the prevention of PI animals (24, 38).

In the 1980's, molecular technology provided the ability to determine the genomic nucleotide sequence of BVDV which led to the development of monoclonal antibody and nucleic acid derived diagnostics for the detection of BVDV (24, 48). Most monoclonal antibodies were produced to neutralize the E2 protein of BVDV which is the major virion envelope protein (18, 24). These antibodies were used as reagents in protein studies that

led to the sequencing of the two biotypes of BVDV, cytopathic and noncytopathic (19, 24, 57). Comparative analysis of the two BVDV biotypes revealed the cytopathic strain produced one additional nonstructural protein (NS-3) in cell culture that was not present in the infected cells of the noncytopathic BVDV strain (19, 24, 57). With further investigation, the cytopathic NS-3 protein was found to be a smaller version of the larger nonstructural protein, NS2-3, present in the noncytopathic strain. The expression of this cytopathic nonstructural protein was thought to occur by either post-translational cleavage of the NS2-3 protein of the noncytopathic strain or, through genetic deletions or duplications of the cytopathic genome (24, 47).

In the 1990's, BVDV was further categorized into two genotypes due to the emergence of new BVDV isolates in the United States and Canada (24). BVDV type I consisted of the early strains of BVDV, while type II BVDV presented similar to the acute severe BVDV with hemorrhagic syndrome (24, 55, 61). Clinical signs of BVDV type II were respiratory disease and diarrhea in all age groups as well as abortion in adult cattle (13, 24). Gastrointestinal lesions seen at necropsy of animals infected with BVDV type II were characteristic of mucosal disease (24).

With the emergence of BVDV type II, there was a renewed interest in developing diagnostic techniques to accurately identify both BVDV genotypes (24). The currently available monoclonal antibodies were tested for cross reactivity with BVDV type II strains and new antibodies were developed to specifically target the type II strains (24). Most of the available type I monoclonal antibodies could not effectively detect the type II strains except for a couple of antibodies against the E2 region (24). Several monoclonal antibody tests were developed during this time, including competitive and capture

enzyme-linked immunosorbent assays (ELISA), monoclonal antibody-based immunohistochemical method, and flow cytometry (24). Reverse transcription polymerase chain reaction (RT-PCR) assays were also introduced and proved to be as sensitive as the monoclonal antibody-based diagnostic tests (24).

In addition, vaccine manufacturers made it a priority to include BVDV type II strains in their vaccines (24). The extent to which type I vaccines protected the fetus was a growing concern since type I vaccines showed negligible efficacy in protection against BVDV type II fetal infections (24). A 20 year retrospective review of fetal protection studies was conducted and found that no one vaccine provided complete fetal protection. This study proposed protection using vaccines varied from 33-86% fetal protection (24, 67).

In early 2000, the focus was on the feedlot industries difficulty with respiratory diseases. Bovine respiratory disease produced a high rate of mortality in feeder calves and negatively influenced weight gain performance (24). Martin studied antibody titers to BVDV, infectious bovine rhinotracheitis, parainfluenza -3, bovine respiratory syncytial virus and two mycoplasma strains in a group of calves before and approximately 5 weeks after entering the feedlot to determine whether an associated pattern of risk of respiratory infection was present with these agents (24, 44). In the study, it was found that BVDV was the most consistent factor associated with an elevated risk of respiratory disease and poor performance (24, 44). Shahriar *et al.* conducted studies of chronic pneumonia with polyarthritis cases, both current and retrospective, in feedlot cattle in Canada and suggested that BVDV and *Mycoplasma bovis* worked together to cause the antibiotic resistance seen with this syndrome (24, 63).

The prevalence of BVDV in stocker calves was investigated by Fulton *et al.* (22, 24). Researchers found that BVDV type Ib was the predominant type present in stocker calves (22, 24). At the time, only one vaccine included the type Ib strain (22, 24). This led researchers to suggest implementation of the type Ib strains in those vaccines that did not include it for broader protection against BVDV (22, 24).

The Academy of Veterinary Consultants released a position statement in 2002 which proposed eventual eradication of BVDV from North America (24, 28). It called on bovine veterinarians in both the U.S. and Canada to work toward a comprehensive BVDV control program (24, 28). Testing and vaccination would be the cornerstone in control programs to quickly identify persistently infected animals and protect susceptible populations through vaccination (24, 28).

Today, researchers and veterinarians are aware of the complex pathogenesis of BVDV and its role in multiple disease processes. Various diagnostic techniques have been implemented to accurately identify PI animals in a period of time which aids in prompt removal of this potent reservoir. Prevention and control programs have been generated to reduce the potential for infection in beef and dairy herds. Vaccines are used in tandem with biosecurity measures to control the transmission of BVDV and the production of acute and PI infections. While much of the pathogenesis of BVDV is clearly understood, this disease still eludes practioners with its rapid ability to mutate and diversify.

Characteristics of Bovine Viral Diarrhea Virus

The BVDV genome consists of approximately 12,308 base pairs (36, 41). The virion is enveloped and spherical in shape with a diameter of 40-60 nm (36, 41). Being an RNA virus, BVDV has a high frequency of transmissible change in its genome through point mutations, RNA recombination and a lack of proofreading (2, 51). The mutation rate from point mutations alone is 10^{-4} per base site, meaning a point mutation can occur with every cycle of viral replication (2, 51). With the capacity for rapid viral replication, the potential for many different mutants increases exponentially and selection for those mutants best adapted to their specific host leads to a higher probability of viral infection (2). The enormous pool of BVDV variants produced by the point mutations proves to be of great advantage to the virus when dealing with the host immune system (2). RNA recombination is another component that contributes to the diversity of BVDV allowing the virus to remain virulent to cattle and cause problems for veterinarians trying to control dissemination of the virus. RNA recombination takes place when two viral genomes within the same cell cross-over at any location along the genome resulting in a new "hybrid" RNA genome which has a completely different genetic and antigenic composition (2, 30). The diversity of BVDV by point mutations and RNA recombination is important to its success and resistance to eradication.

BVDV Bioptyes:

BVDV is categorized into two biotypes: cytopathic and noncytopathic. The distinction between the biotypes is determined by their effect on cell culture and whether

infected cells are lysed, as occurs with the cytopathic biotype, or remain unaltered, as in the noncytopathic biotype (30). The cytopathic biotype is not as resilient *in vivo* due to rapid immunological clearance by a healthy, immunocompetent animal when compared to the noncytopathic biotype (30, 56). Research conducted on cultured bovine macrophages showed prompt stimulation of innate immune effectors by the cells infected with the cytopathic biotype, while no response was elicited from cells infected with the noncytopathic biotype (56). The cytopathic biotype is also limited in its' tissue distribution to the gastrointestinal, mucosal and submucosal tissues (5, 30). It is the general understanding that the cytopathic biotype results from a recombination event in the noncytopathic biotype (30). The cytopathic biotype is therefore self-limiting in nature since it induces cell apoptosis and prompt stimulation of the immune system (30, 31). In the persistently infected animal, death can result if the cytopathic BVDV strain is antigenically similar to the existing noncytopathic strain of the infected host resulting in mucosal disease (4, 24).

Unlike the cytopathic biotype, the noncytopathic biotype can produce both acute and persistent infection (49). Persistent infection of the fetus occurs by acute infection of a pregnant dam between 18 and 125 days of gestation (25). The noncytopathic biotype also has wider tissue tropism, including lymphoid tissues, the mucosa of the lower digestive tract and respiratory tract in acute infections and a widespread distribution of lymphoid tissues, respiratory tract and the upper and lower digestive tracts in persistent infections (39). In addition, the noncytopathic biotype causes immunosuppression by depletion of lymphoid cells in acute infections (5). It is also the key source for maintaining long term presence of the virus in the environment (2).

BVDV Genotypes:

Bovine viral diarrhea virus is further classified into two genotypes, type I and type II, that differ by their genetic sequences (36). There is approximately 60% homology between the nucleotide sequence of the two BVDV genotypes (2). The important distinction between the two genotypes is their antigenic diversity. BVDV type I isolates "comprise most classical, non-hypervirulent BVDV strains" while the type II isolates include strains from hypervirulent outbreaks in North America as well as some atypical and "low or moderately virulent strains of BVDV" (30). However, the vast "majority of both type I and type II BVDV isolates are of low virulence and induce subclinical to very mild disease" as reported by Bolin and Grooms (2). The genotypes are further divided into subgenotypes, however, it is believed there is approximately 80-85% homology between the thirteen BVDV subgenotypes, with BVDV type I consisting of eleven subgenotypes and BVDV type II having only two subgenotypes (2). Typically, BVDV type I is correlated with "persistent infections, congenital defects, and weak calves, while type II BVDV isolates are more commonly found in aborted fetuses" (2). The presence of BVDV genotypes and their subgenotypes further demonstrates the heterogeneity and versatility of this complex *Pestivirus.*

Persistent infection:

Persistent infection (PI) is defined as fetal immunotolerance for the BVDV strain that infects the fetus from 30 to 125 days of gestation (56). PI calves can be clinically normal, or have a variety of clinical signs including low birth weight, stunted growth,

immunosuppression, respiratory disease, or simply a poor performer (1). Persistently infected calves shed abundant noncytopathic virus, which can readily transmit to herdmates (39). Virus transmission from PI calves is much more effective than transmission from cattle acutely infected with BVDV (39). Acute infections by non-cytopathic BVDV typically cause subclinical disease and a subsequent increase in antibodies to BVDV (39). Pregnant dams acutely infected by noncytopathic BVDV early in gestation have the potential to produce a persistently infected offspring (39). Therefore, understanding the pathogenesis of BVDV is important in diagnosing persistent infection of calves in a herd.

Worldwide, 60 to 85% of cattle have been exposed to BVDV and 1 to 2% are considered persistently infected (51). This PI prevalence rate is thought to be underestimated, since many persistently infected animals are poor-doers or unthrifty and are culled before a diagnosis is determined (1, 65). Consequently, only PI animals that survive long enough for testing are taken into account and the chances of a PI calf surviving beyond two years is unlikely with a majority succumbing to mucosal disease (1). Since there is such a high mortality rate among PI animals, a higher prevalence rate of PI animals is not seen with BVDV (1).

Persistently infected animals have a major economic impact on the cattle industry. It is estimated that the U.S. suffers an approximately $3 billion dollar loss to BVDV each year and numbers are increasing (51). Cattle are sorted into weight groups when sold to cattle producers and feedlot operators. Selection preferences for groups of lower weight weaned calves could result in the purchasing of several PI animals to introduce to a new group of animals compared with heavier weight weaned cattle (65).

Diagnosis of BVDV

The diagnosis of persistently infected animals is the cornerstone of BVDV control programs. Several diagnostic tests are available to determine persistent infection status of a herd as well as acute BVDV infections. Certain tests do have advantages over others depending on producer objectives (i.e. whole herd screening or disease outbreak) and goals (34). Therefore, the accuracy of the diagnostic testing used is crucial for the detection of BVDV.

Virus Detection:

Virus isolation. Virus isolation (VI) detects live BVDV. Cell monolayers (Madin Darby bovine kidney (MDBK) cells, bovine turbinate (BT) cells or bovine testicular cells) are inoculated with the test specimen(s), followed by either immunofluorescence or an immunoperoxidase plate assay using BVDV specific antibodies to detect the virus after 3-5 days of incubation (8, 41, 62). Appropriate antemortum test specimens include whole blood, "serum, nasal swabs, feces, semen and various tissues" (8, 20, 62). The buffy coat, extracted from whole blood, is the sample of choice, especially in the identification of acute infections (8, 20, 62). The appropriate test specimens from necropsy or an aborted fetus are "lymphoid organs such as spleen, Peyer's patches from the small intestine, mesenteric lymph nodes, and thymus" (62). The advantages of the immunoperoxidase plate assay compared to the immunofluorescence detection system are 1) the capacity to process large numbers of samples at a time and 2)

a fluorescent microscope is not necessary to detect the BVDV antigen and interpret the results (8). The virus isolation diagnostic technique has the capability of quantifying the amount of virus in samples through titration (62). Virus isolation is considered the most reliable viral reference test for the detection of BVDV (24, 34, 62).

Disadvantages of virus isolation include 1) frequent requirement for a second passage in cell culture to eliminate non-specific antibody binding; 2) the need for specialized equipment and techniques to maintain cell cultures; 3) increased time, labor and expense when performed on individual samples (20, 37, 62). In addition, calves younger than three months of age should not be tested with VI due to the presence of maternal antibodies which can interfer with virus growth and result in a false negative result (62). If this technique is used on young calves, retesting would be necessary (62). Quality control is also important with virus isolation as contaminants of the cell culture or test samples can render a false negative or false positive result (34, 41). Also, there are numerous protocols for the virus isolation technique which can lead to inconsistencies among diagnostic laboratory results (20).

Enzyme-linked immunosorbent assays (ELISA). Antigen detection using ELISA offers a rapid, inexpensive alternative to virus isolation with a high sensitivity (100%) and specificity (98.4%) for BVDV (16, 20, 34, 41). The assay produces a color reaction which is measured using optical density values compared to a negative control sample (34). The optical density values may be used as a semi-quantitative measure of BVDV antigen (34). Cell culture training and facilities are not necessary to run ELISAs (34). This technique is a useful screening tool in the detection of PI animals; however, it is not reliable for diagnosing acute infections (62).

Immunohistochemistry (IHC). Immunohistochemistry uses a monoclonal antibody (15C5, which reacts with the E0 protein, a highly conserved envelope protein in BVDV strains) to detect BVDV in formalin-fixed, paraffin-embedded specimens (8). Skin biopsies, particularly of the ear, are commonly used for their ease of sample collection and transport (34). The viral antigen has proven to be stable in the formalin-fixed skin and can be detected in tissue stored in formalin for as long as one month or stored unfixed in a refrigerator for ten days before the possibility of a false negative result (20, 43). This method is very popular for herd testing especially since the virus in skin biopsies is not affected by maternal antibodies (27). IHC on ear notch samples has proven to be an accurate tool for detecting persistent infection in cattle with a sensitivity of 100% and a specificity of 98.8% (16, 20, 21). However, several studies have shown the possibility of acute infections being detected by IHC. Discrepancies exist among studies regarding whether the density and distribution of viral staining between acute and persistent infections are distinct enough to differentiate between the two disease states. Njaa *et al.* described the IHC viral staining of acutely infected animals as nonexistent to irregular "small, discrete foci distinguishable from the extensive staining seen in a PI animal" (50). Also, Liebler-Tenorio *et al.* described the distribution of viral antigen staining in acute infections as different and limited to certain tissues when compared to the staining seen in the tissue of a persistently infected animal (39). In this study, the distribution of BVDV antigen from acute infections cleared from most tissues after thirteen days post inoculation (39). However, Cornish *et al.* noted the detection of viral antigen distribution and intensity in acutely infected animals was indistinguishable from the staining of PI animals (16). While the distribution and intensity of staining decreased

over time, it proved that more than one diagnostic detection method should be used in a BVDV control program (16).

Disadvantages of IHC are it is labor intensive and requires laboratory facilities for sample processing that can take 3-5 days (16, 20, 37). With its' multi-step protocol there is room for technical error (20). A subjective component to the microscopic analysis of the samples exists leading to variability in results among laboratories and technicians (20). In addition, the possibility of detecting acute infections when using IHC requires the need for follow-up testing with a more specific diagnostic test to distinguish acute infection from persistent infections (16, 27).

Nucleic acid-based Detection Methods

Reverse Transcription Polymerase Chain Reaction (RT-PCR). Selected specific nucleotide sequences of the BVDV genome can be amplified using reverse-transcriptase PCR (RT-PCR). This technology involves the "binding of specific DNA oligonucleotides to cDNA target sequences resulting in amplification of DNA fragments" which are detectable using gel electrophoresis or fluorgenic probes (62). This technique is highly sensitive (100%) and has the ability to detect small quantities of viral RNA regardless of the animal's age or presence of neutralizing antibody (17, 34, 62). The possible test samples are numerous including bulk milk samples, serum or plasma samples, buffy coat cells, whole blood, semen and ear-notch samples (20, 34). Formalin fixed tissues are not ideal samples for PCR because the fixed genetic material is extensively fragmented and there is a risk of false negatives when using this type of specimen (62). RT-PCR is cost effective when used on pooled or bulk samples and

typically has a quick turnaround time of less than two days (20, 37). However, the main disadvantage to testing pooled samples is that RT-PCR detects all types of BVDV whether it's an acute infection, persistent infection or vaccination with a modified live BVDV vaccine (37, 62). In this circumstance, a follow-up screening test such as virus isolation, antigen capture ELISA or IHC would need to be used on all the animals in that particular pool to find the affected animal (37).

Protocols for RT-PCR involve a multiple step process that is time consuming and susceptible to processing error. The risk of contamination at any point in the protocol is high and can lead to variable results (20). The efficacy of PCR is dependent on the selection of primers used to bind the genetic material of BVDV isolates in the field (2, 20). RT-PCR has the ability to distinguish between genotypes of BVDV if the appropriate primers are selected (37, 41, 62).

In conclusion, diagnostic tests for BVDV detection need to be accurate in order to facilitate the veterinarian and producer goals for the herd (16). The sensitivity and specificity of several diagnostic tests have been compared in recent studies. Deregt *et al.* compared RT-PCR and direct PCR without reverse transcription to VI and found 100% sensitivity between the two assays (17). Cornish *et al.* compared IHC and antigen-capture ELISA (AgELISA) to the gold standard test of VI and RT-PCR. Both IHC and AgELISA detected 100% of the PI calves in the sample group (16). However, these tests also detected acutely infected calves resulting in a specificity of 98.8% for IHC and 98.4% for AgELISA, respectively (16). The detection of acute BVDV infections using IHC and AgELISA in this study continued from several months after the initial herd

screening (16). Researchers recommended re-testing all IHC and AgELISA positive animals with VI or RT-PCR using buffy coat samples 30 days after the initial test (16).

Prevalence of BVDV

Research suggests that the presence of one or more PI animals within a cattle herd is the single most important way to maintain BVDV infection (68). The BVDV noncytopathic biotype is highly adapted to the bovine host allowing it to persist in the environment and maintain a low level of virulence (2). The ability to cause minimal adverse effects and allow survival of the host means BVDV achieves significant shedding potential in order to improve further viral transmission (2). The prevalence of BVDV in cattle herds is measured by testing for persistently infected animals. In the 1980's, Bolin reported a 1.7% PI prevalence in 66 beef herds tested (3). In 1988, Howard reported 0.78% prevalence of adult cows in AI centers in the Northeastern U.S. (32). During the 1990's, prevalence studies were performed on dairy cattle in California and Michigan showing a 0.5% and 0.13% prevalence rate, respectively (33, 49). A Canadian prevalence study in the early 1990's found a PI prevalence of 0.17% in a feedlot in Western Canada (66). Prevalence studies have become very popular during this decade with studies focusing on dairy, beef and feedlot populations in mostly the central and western parts of the U.S. The majority of PI prevalence rate results were less than 0.6% across the different populations this decade so far; the exceptions were a 10.5% PI prevalence rate of beef neonates in Wyoming and a 1.8% PI prevalence of dairy neonates

in Michigan (16, 27). In comparison with European studies, the U.S. shows a lower prevalence rate of PI animals (24). This is evidence of the low virulence BVDV maintains in cattle herds and how its mechanism of action is so successful in ensuring its ability to infect large populations.

Prevention of BVDV

The key objectives of BVDV control programs in the U.S. are 1) identify and remove PI cattle quickly to eliminate the primary source of viral dissemination, 2) on-going herd surveillance, 3) implement biosecurity protocols, and 4) immunization strategies (20, 42, 64). Herd screenings using diagnostic testing identifies those cattle with acute or persistent infections with BVDV and allows prompt isolation or removal of infected animals from the general herd (20). Typically, more than one diagnostic test should be used on positive animals in the event of an acute infection (20). Once eradication of PI animals is achieved, herd surveillance should be implemented to maintain a BVDV-free status (34). Successful biosecurity practices are important in preventing the reintroduction of BVDV especially once a BVDV-free status is attained (64). Eliminating contact with other animals of unknown BVDV status such as through fence line contact, travelling to cattle shows, or the acquisition of new additions to the herd is imperative (64). These animals should be quarantined for 2-3 weeks to test PI status if it's a new addition or monitor for sickness if commingling with other animals has occurred (64). It is critical to separate all potentially infectious animals from the pregnant cows in the herd, especially those in early gestation which if acutely infected

could result in intrauterine infection of the fetus (64). New pregnant additions to the herd should be quarantined, as well as prohibited from calving in the same area as the established herd (64). In addition, indirect exposure through fomites, contaminated feed/water troughs and clothing are important prevention points to consider when implementing biosecurity protocols (64).

Vaccine protocols are used to give additional protection against acute and persistent infection but should not be used as the sole means of BVDV prevention (54). The types of BVDV vaccines available are live and inactivated vaccines (36). The live vaccines are derived from attenuated strains of BVDV and generally provide a longer duration of immunity compared to inactivated vaccines (36). The inactivated vaccines are safe and incorporate an adjuvant that stimulates an adequate immune response (36). However, this response may not be superior to that of the live vaccines (36). Ideal vaccines to use in any biosecurity program are those that can successfully stimulate the immune system and respond if challenged, cross protect against genotypes and subgenotypes of BVDV and offer some amount of fetal protection (26). Vaccines are not 100% efficacious and vaccine failure as well as immunologic failure can occur making reliance on vaccines alone a risky practice (30).

STATEMENT OF RESEARCH OBJECTIVES

This study was designed to investigate the prevalence rate of persistently infected stocker calves with BVDV in the Southeastern US, as well as determine whether one specific weight group had a higher incidence of persistent infection. Our hypothesis was that lower weight cattle had a higher incidence of persistent infection compared to heavier weight cattle. In addition, suggestions that stocker cattle from the Southeast have increased disease rates associated with BVDV-PI were investigated and compared with prevalence rates of persistent infections in other regions of the U.S.

CHAPTER III

MATERIALS AND METHODS

Calves: A total of 7,544 yearling stocker calves were sampled for the presence of BVDV from March to December 2005. The calves were purchased by an order buyer from local auction markets located in the Southeast region (AL, FL, GA, MS, and TN) of the United States. Following purchase, they were transported to a central holding facility where the calves were processed, sorted and assembled into truckload lots based on their average weights. The cattle were grouped based on average truckload weights into groups to determine the number of animals positive for PI-BVDV. The average weight within a truckload group did not vary by more than 50 pounds. Ear notch biopsies were collected at the processing facility and then shipped to the laboratory and processed for further analysis.

Specimens: Triangular shaped, 1-2 cm long skin biopsy specimens (ear notches) were collected from the ventral margin of the left or right pinna using commercial hog notch pliers. The specimens were collected post-sorting, during vaccination administration and processing, when the animal was restrained in a chute. All tissue specimens were immediately immersion-fixed in zinc sulfate formalin (Z-fix, Anatech, hereafter referred to as zinc formalin) following collection and shipped to the laboratory on a weekly basis.

Specimens were sent either pooled together in plastic bags containing zinc formalin or in individual tubes of zinc formalin along with the average weight lot information for the truckload. Specimens were processed promptly upon arrival at the laboratory.

Tissue sampling and processing: A full-thickness sample of each specimen was trimmed and placed into one compartment of a six compartment tissue embedding cassette (five separate specimens per cassette plus one compartment with a non-relevant tissue (kidney) for orientation purposes). The samples were then rinsed in cool running tap water for approximately 10-20 minutes to remove sulfate ions and thus prevent precipitates from forming in the tissue spaces thereby resulting in inadequate staining and analysis. The tissue cassettes were rinsed then placed in 70% alcohol overnight and processed the next morning by the procedure described by Haines *et al*. (29) using a Shandon Excelsior automated tissue processor *(Waltham, MA).* The tissues were passed through a series of graduated alcohol suspensions for dehydration and then a clearing solution of xylene to remove the dehydration agents. Following the processing, ear notch tissues were embedded in paraffin blocks and histological sections were cut at 4 micrometers. Sections were mounted on silane-coated glass microscope slides and dried in a 58º oven for 30 minutes prior to BVDV immunohistochemisty.

Immunohistochemistry: Immunohistochemistry (IHC) was performed for the detection of BVDV antigen in specimens of skin, collected by ear notch using an automated slide processing system (29). The monoclonal anti-BVDV primary antibody, 15C5 (Ed Dubovi, Cornell University) was used at a dilution of 1:1500 for viral antigen detection.

Biotinylated goat anti-mouse IgG detected by Streptavidinhorseradish peroxidase (LSAB2 Kit, DAKO Corporation, Carpinteria, CA) and Nova Red Substrate (Vector Laboratories, Burlingame, CA) were used to detect antigen and develop the color reaction. Quality control was done for each batch of samples processed. Positive control consisted of a slide containing a tissue sample from a PI animal known to contain BVDV by repeated virus isolation. Negative control was a duplicate sample slide processed in the absence of primary antibody, but reacted with all other IHC agents.

Examination of the stained slides was performed using a light microscope (*Zeiss, Gottingen, Germany*) at 10X magnification for tissue scanning and 40X magnification for investigation of suspect areas within the tissue. Each slide was approached in the same manner by first checking the positive and negative control tissues for accuracy of staining and processing. Then, the ear notch sample was analyzed thoroughly for evidence of viral staining in the tissue epidermis and hair follicle epithelium. Evidence of BVDV reactivity was graded as positive or negative. Positive staining was considered characteristic of staining for PI animals and was determined to represent a PI animal, Figure 1B.

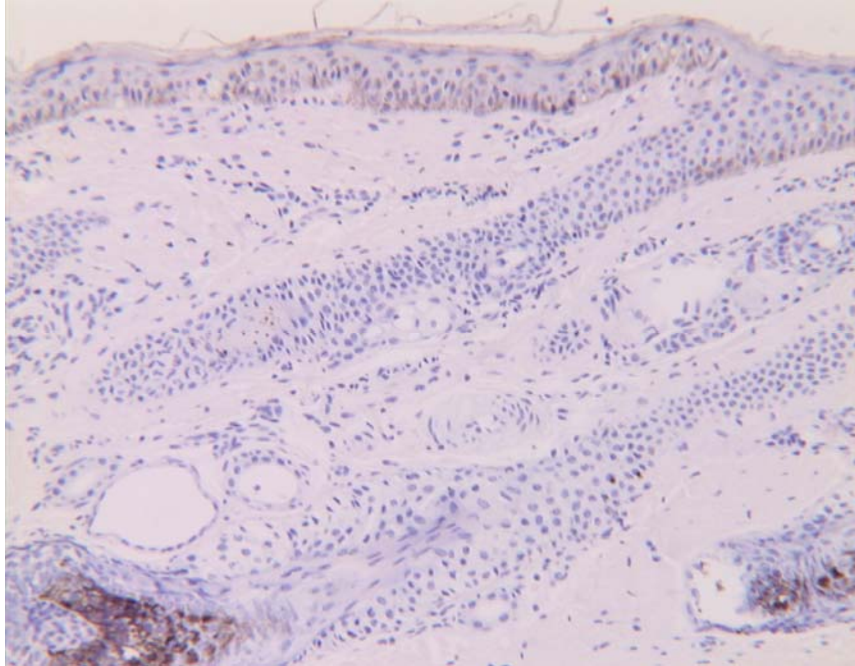

Figure 1A. Negative control of skin biopsy from ear tissue.

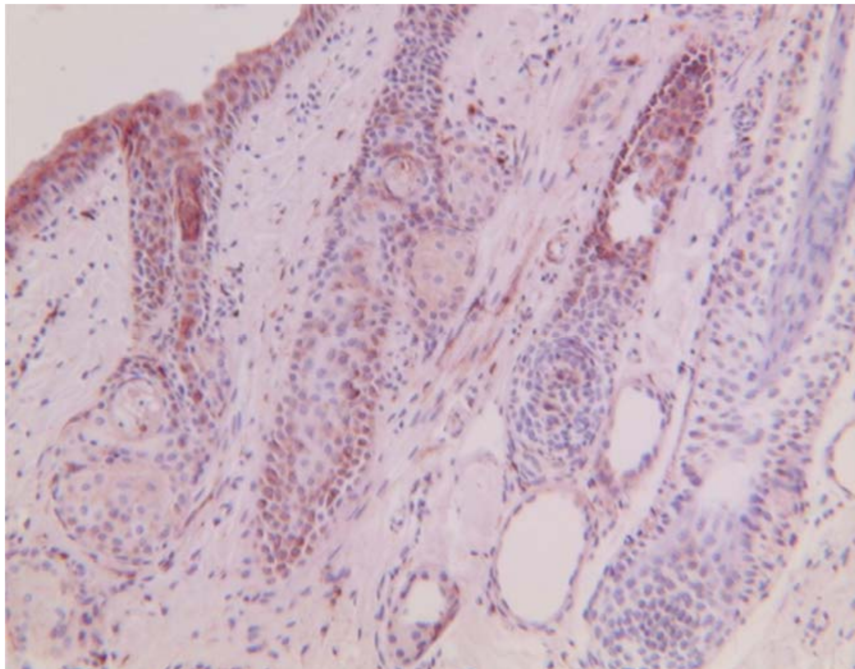

Figure 1B. BVDV positive skin biopsy. Immunohistochemical staining for bovine viral diarrhea virus antigen was present in the epidermis, root sheath epithelium and hair bulb.

Statistical Analysis: Data were analyzed using the Statistical Analysis System version 9.1 (SAS Institute, Cary, NC). The procedure of frequency (PROC FREQ) was used to determine the proportion of occurrence of BVDV PI animals (prevalence) for groups of animals by weight. Differences in the prevalence of BVDV PI animals were analyzed with a chi-square test. The logistic procedure (PROC LOGISTIC) was used to investigate the relationship between the discrete response (BVDV positivity) and the explanatory variable (weight). The odds ratio estimate and its respective confidence interval were computed along with the parameter estimate based on the maximum likelihood function.

CHAPTER IV

RESULTS

Microscopic examination of immunohistochemistry staining of all submitted ear notch tissue samples from stocker calf samples revealed that 24 test samples out of 7,544 test samples were positive for BVDV-specific antigen staining, Table 1. Therefore, the overall PI prevalence rate was determined to be 0.318%. Further analysis of positive test samples was done according to weight groups through a chi-square test, Table 2.

To determine statistical significance, the procedure of frequency (PROC FREQ) was used to determine the proportion of occurrence of positive BVDV diagnosis (prevalence) for both groups of animals (<400 and ≥ 400 lbs). Differences in the prevalence of BVDV PI animals were analyzed through chi-square test. The logistic procedure (PROC LOGISTIC) was used to investigate the relationship between the discrete response (BVDV positivity) and the explanatory variable (weight). The odds ratio estimate and its respective confidence interval were computed along with the parameter estimate based on the maximum likelihood function and are summarized in Table 3.

Table 1. Results of immunohistochemistry testing for BVDV antigen categorized by the average weight class. The number of animals and the results of IHC testing are given by the average weight category.

AVG WEIGHT (lbs.)	**# animals**	**# IHC positive**
175	21	0
200	540	2
240	233	1
250	903	3
275	89	0
280	119	0
300	1084	7
325	188	1
330	78	0
340	252	3
350	498	1
365	196	1
375	49	0
395	110	0
400	386	1
450	477	1
460	98	0
475	182	1
493	83	0
500	771	1
520	110	0
525	247	0
530	115	0
550	29	0
600	327	0
630	176	1
640	115	0
650	68	0
TOTALS	7,544	24
Prevalence rate	24 / 7,544	0.318 %

Table 2. Distribution of BVDV PI positive test samples between sorted weight groups. The weight groups represent the average weight of animals sorted in individual truckload lots at the point of sample collection.

Weight Groups (lbs.)	Number of Animals sampled	Number of animals Positive for BVDV	PREVALENCE rate (%)
150-299	1,905	6	0.315
300-399	2,455	13	0.529*
400-499	1,226	3	0.244
500-599	1,272	1	0.078
600-699	686	1	0.146
NET TOTAL:	7,544	24	0.318

**P*-value <0.05

Table 3. Summary of statistical analysis of weight groups with break point at 400 lbs. for prevalence of BVDV PI animals.

Weight Group	PI prevalence	P-value	Odds ratio	Odds Confidence Interval
<400 lbs.	0.44 %	0.0337	2.782	1.038-7.459
≥400 lbs.	0.16 %			

CHAPTER V

DISCUSSION

Results of immunohistochemistry testing indicated a PI prevalence of 0.318 % in a sample size of 7,544 beef cattle. Statistical analysis revealed that the overall prevalence was estimated as 0.32% with a ± 0.13% standard error. Therefore, at a 95% confidence interval, the prevalence is between 0.19% to 0.45%. Statistical analysis supports that the prevalence rate is significantly less than 1.0%. From this sampling of stocker cattle chosen by an order buyer from auctions in Alabama, Florida, Georgia, Mississippi and Tennessee which represents multiple auction sites in the Southeast, it can be concluded that the prevalence of PI animals in the Southeast is approximately 0.3%. The prevalence rate determined in this study was from randomly collected samples and did not incorporate bias due to the collection of samples from animals suspected for BVDV infection or re-tests of animals identified in a BVDV herd screening effort. This bias can be commonly introduced in diagnostic laboratory test reporting as sample submissions often do not accurately reflect infection rates in the overall population. The results of the present study reflect the prevalence rate in stocker cattle moving through regional (southeast USA) auction markets.

In this study, immunohistochemistry was performed to detect persistent infection with BVDV. It is recognized that immunohistochemistry primarily detects persistent

BVDV infection (39, 50). Based on previous studies, there may be a small proportion of acute BVDV infections that can be detected by immunochemistry (16). However, the intensity of antigen staining and the distribution of antigen is significantly less in acute BVDV infections. Staining observed in all immunohistochemistry positive test samples in this study were indicative of BVDV persistent infection based on the distribution and intensity of staining.

The results of this study indicate that there has been a misconception that the Southeast have a higher prevalence of PI animals than other areas of the United States. The 0.318% prevalence rate reported from this study compared closely, if not less than other rates reported from the northeast, central and Midwestern states. Lonergan *et al.* reported the same 0.3% rate in cattle from southwest Kansas and the Texas panhandle (43). Fulton *et al.* reported prevalence estimates of PI cattle from Oklahoma and Texas to be 0.6% (23). In one study from 1990, Howard *et al.* conducted a prevalence study of reproductively mature cattle at artificial insemination centers in the northeastern U.S. and found a 0.78% prevalence rate (32). It is important to note that the cattle tested in this study were adult cattle and do not coincide with the age of cattle tested in the present study. According to Dr. Bruce Brodersen (per personal conversation), University of Nebraska has processed over 1 million ear notch samples and found a percent positive of 0.6%. The test results reported at University of Nebraska does incorporate re-testing of duplicate sampling and the inclusion of test samples from suspect cases of BVDV infected animals. The percentage of positive animals reported from Nebraska is not representative of a random test sample. Therefore, it is concluded that no one region of

the U.S. has a higher incidence of PI animals than another judging from the similar PI prevalence rates reported from the different regions.

Analysis of the positive test samples between weight groups was done according to the prescribed sorted weight groups, Table 1. Statistical analysis by weight group was difficult due to potential bias introduced during sorting and would have benefited from a larger test sample size within each weight group. Animals with <400 lbs of weight showed a significantly higher ($P < 0.05$) BVDV PI prevalence (0.44%) compared with animals with ≥400 lbs of weight (0.16%). The difference in the BVDV prevalence of 0.28 % between the two groups was statistically significant, with a two sided P-value = 0.03. The comparative prevalence difference between the two populations as used may not be the most accurate method for comparison. An alternative of comparing proportions is to compare their corresponding odds. This is used for regression models of binary data (positive vs. negative). The odds of identifying a PI animal in a population of calves with a weight < 400 lbs was estimated to be 2.78 times greater than in a population with a weight ≥400 lbs (95% confidence interval: 1.03 times to 7.45 times). There were almost three PI animals in the group with <400 lbs, for each PI animal in the group ≥400 lbs. The odds ratio is the only parameter that can be used to compare two groups of binary response outcomes (positive vs negative) from a retrospective study. However, conclusions must be made carefully when interpreting odds ratio. This was an observational study (not random experiment) so that a cause-effect conclusion cannot be established. From the analysis it can be concluded that there is an association between weight <400 lbs and the odds of diagnosing BVDV PI from ear notches samples by immunohistochemistry. In addition the scope of inference is limited to the sampled

population (AL, FL, GA, MS and TN states), so it can not be extrapolated to all the bovine populations.

Analysis of prevalence rates can be difficult due to the strong herd influence that impacts infection rates. The possibility of a "clustering effect" may exist, meaning if one herd has a PI detected, it is likely more than one PI animal exists in that same herd (43). This increases the immunity of herdmates and when co-mingling occurs in the feedlot situation, those naïve animals that are introduced and exposed to the PI are affected resulting in high morbidity rates in the feedlot (43).

As with any test sampling, this study was susceptible to bias and confounding factors within its design. The preferential selection of a certain type of cattle by the order buyer at auction locations may be one potential bias which influenced true random sample selection. In addition, the cutoff for sorting and grouping cattle in truckload lots must be considered when analyzing the results. The possibility of misclassification of animals upon grouping all must be considered when analyzing these results. However, cattle were selected before disease and exposure status was known decreasing the selection bias by the order buyer. Also, the population of cattle studied was not completely randomized but correlated to the reality of the cattle marketing process. The retesting of positive animals to confirm persistent infection was not possible leading to scrutiny of whether transient infection was responsible for the positive result. However, IHC positive samples are rarely due to acute or transient infections and in most circumstances represent PI infection.

In conclusion, the prevalence of PI-BVDV calves in the Southeastern USA is estimated at 0.3%. The hypothesis that lower weight cattle are more likely to be

persistently infected was confirmed when comparing the <400 and ≥400 lbs. weight groups. Calves that are <400 lbs. are 2.78 times more likely to be persistently infected than the calves in the ≥400 lbs. weight group. Finally, by comparing the 0.318% PI prevalence rate found in this study with the PI prevalence rates of other regions in the United States, it is determined that calves in the Southeast do not necessarily have a higher disease rate from persistent infection of BVDV.

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