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SEROLOGICAL EVALUATION OF FIVE UNVACCINATED HEIFERS FOR DETECTING HERDS WITH CATTLE PERSISTENTLY INFECTED WITH BOVINE VIRAL DIARRHEA VIRUS

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ABSTRACT

SEROLOGICAL EVALUATION OF FIVE UNVACCINATED HEIFERS TO DETECT HERDS WITH CATTLE PERSISTENTLY INFECTED WITH BOVINE VIRAL DIARRHEA VIRUS

By

Roxanne Bee Pillars

A key to eradicating bovine viral diarrhea virus (BVDV) is the identification and removal of all BVDV persistently infected (PI) cattle. This requires testing all cattle within a herd, a costly and labor intensive endeavor for the producer. It would be helpful if herds containing PI cattle could be identified prior to committing the resources necessary for whole herd screening. The objective of this study was to determine if the evaluation of BVDV antibody titers in five unvaccinated heifers was an accurate method to predict if a herd was infected with BVDV.

Blood samples were collected from all cattle in 14 dairy herds. Virus neutralizing antibody titers to type I and type II BVDV were determined on five randomly selected unvaccinated heifers 6-12 months of age from each herd. A herd was classified as likely to contain PI cattle if at least three heifers had antibody titers $\geq$ 128. Virus isolation was performed on all cattle to identify PI animals and definitively classify herds as infected or uninfected.

PI cattle were identified in six herds. Sensitivity and specificity of the serological evaluation of five heifers for identifying these herds was 66% and 100% respectively. In herds containing PI cattle, the predominate BVDV titers in the sampled heifers corresponded to the genotype of the isolated virus.

DEDICATION

It gives me great honor to dedicate this work to my parents,
Claude and Mary Jo Pillars.
Thank you for your unfailing love, support, and
encouragement, even while not completely understanding
why I do the things that I do.

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INTRODUCTION

Worldwide, bovine viral diarrhea virus (BVDV) is one of the most costly viral diseases in cattle, resulting in an estimated annual loss of $10-40 million per million calvings [1]. The complex nature of the virus and its many clinical manifestations has made control and prevention of BVDV extremely challenging. BVDV is particularly difficult to control because fetal infection prior to the 125th day of gestation can result in immunotolerance and the birth of calves persistently infected (PI) with BVDV [2]. These PI cattle continuously shed large amounts of BVDV throughout their lives, and are the primary reservoir for infection within and between herds [3-6]. Thus, effective control and prevention of BVDV is dependent on the identification and removal of all PI cattle from a herd.

The overall prevalence of PI cattle is very low, ranging from 0.1-1.8% in various [7-15]. Moreover, many PI cattle appear clinically normal [2]. Therefore, the identification of PI cattle requires individual testing of all cattle in a herd for BVDV, which is expensive and labor intensive for the producer. In short, identifying PI cattle is like looking for the proverbial needle in the haystack. It would be nice to know in which haystack to look.

Various strategies have been proposed to identify herds containing PI cattle. Determination of BVDV antibody in bulk tank milk samples can accurately identify herds infected with BVDV, and is part of the national eradication programs in many European countries where vaccination for BVDV is prohibited [16-19]. However, this method is not of practical use in the United States due to the widespread use of BVDV

vaccines in lactating cows. Detection of BVDV RNA in bulk tank milk samples by polymerase chain reaction (PCR) has been demonstrated, but its usefulness as a herd screening test is not well documented [20, 21]. A major disadvantage of both of these methods is they only screen lactating cattle; the results cannot be extrapolated to the rest of the herd.

Serological evaluation of young, unvaccinated heifers has been shown to accurately identify herds with PI cattle [22, 23]. The basis for this strategy is that a high prevalence of seropositive animals in a herd is indirect evidence that a PI animal exists in that herd [12]. Young heifers essentially serve as sentinels for detecting circulating BVDV, which is generally the result of a PI herd mate. The advantage of this method over the bulk tank tests is that it is more representative of the entire herd. The probability of a PI animal being present in a herd was 0.99 if three of five heifers had BVDV neutralizing antibody titers $\geq$ 128. Conversely, if three of five heifers had titers $\leq$ 64, the probability of the herd containing a PI animal was < 0.01 [23]. These probabilities, however, were calculated for herds in which all cattle had been tested for both BVDV and BVDV antibodies prior to the study [23]. This technique has not been validated in herds with unknown BVDV status.

The purpose of this study was to validate the serological evaluation of five unvaccinated heifers 6-12 months of age as an accurate method for identifying herds with BVDV PI cattle.

CHAPTER 1

LITERATURE REVIEW

Introduction

Bovine viral diarrhea virus (BVDV) is one of the most important viral diseases of cattle. It was first described in the United States in 1946 associated with epizootics of diarrhea [24]. Since then the virus has been isolated from animals throughout the world. Infection with BVDV may be manifest as several different syndromes including subclinical infection, diarrhea, immunosuppression, and respiratory disease. It has also been identified as the cause of reproductive failure including infertility, early embryonic death (EED), abortions, and congenital defects. More recently BVDV has been associated with outbreaks of a severe hemorrhagic syndrome and acute death [25, 26]. A unique feature of BVDV is that fetal infection prior to the 125^{th} day of gestation can result in immunotolerance and the birth of calves persistently infected (PI) with BVDV [2]. These PI cattle are the major reservoir of BVDV and serve as the primary source of viral spread within and between herds [3-6, 27].

The economic impact of BVDV varies between herds depending on the immune status of the population as well as the pathogenicity of the infecting virus. As a result of this variation, the economic cost of BVDV can only be approximated. Based on a computer spreadsheet model simulating different annual incidence risks of infection, the losses due to BVDV at the population level has been estimated in the range of $10-40 million per million calvings [28].

This review will cover what is currently known about BVDV infection: the virus, the consequences of prenatal and postnatal infection, prevalence and incidence, viral transmission, risk factors, diagnosis, identification of BVDV infected animals and herds, control and prevention.

The virus

Bovine viral diarrhea virus is a positive-sense, single-stranded RNA virus. It belongs to the genus *Pestivirus* of the family Flaviviridae. Isolates of BVDV are further classified based on their biotype and genotype.

Biotype refers to the ability of the virus to cause cell pathology in infected cell cultures. Cytopathic virus destroys cells in culture, while noncytopathic virus causes no visible cell damage. A recent study found that most field isolates were noncytopathic rather than cytopathic. Also, the more virulent strains of BVDV tended to be noncytopathic [29].

Current theory is that cytopathic BVDV evolved as a mutant strain of a noncytopathic virus [30]. Initial BVDV isolates were noncytopathic [2]. As mucosal disease (a fatal form of BVDV infection to be described later) was recognized and studied, cytopathic BVDV was isolated that was antigenically similar to the noncytopathic virus that was also isolated from these cases. Furthermore, only noncytopathic BVDV has been isolated from PI cattle [5, 27]. If PI cattle represent the mechanism by which BVDV persists within the population, then it makes sense that the noncytopathic BVDV must be the normal, or natural, form of the virus, while cytopathic strains are an aberrant form. RNA viruses by nature have poor proofreading mechanisms during genome replication. Thus it is common for base-substitutions (1 error/10,000 base pairs) to occur [31]. Some of these errors, or mutations, will be lethal to the virus, while others will change certain proteins resulting in altered viral characteristics such as cytopathogenicity [31].

With the development of polymerase chain reaction (PCR) in the 1990's, it became possible to differentiate BVDV into two genotypes, type I and type II. Type I BVDV isolates are most commonly associated with subclinical or mild respiratory disease while type II strains tend to be associated with severe disease, including the recently described hemorrhagic syndrome [25, 26, 29]. Most commercial vaccines and diagnostic tests are directed towards type I BVDV isolates [25, 26]. The prevalence of type II BVDV appears to be increasing in North America over the last 20 years, while it remains uncommon in Europe [32, 33]. As a result newer BVDV vaccines now include both type I and type II strains.

Postnatal infection with BVDV

The majority of BVDV infections (70-90%) in susceptible cattle are subclinical [3]. Cattle may develop a mild fever and become leukopenic before developing serum-neutralizing antibodies and clearing the virus from their body. There may be an associated decline in milk production [34], but this is generally very mild and of such short duration, that it often goes unnoticed.

Clinical infection tends to occur in young cattle between six months and two years of age. Following a 5-7 day incubation period, affected cattle develop extremely high fevers and severe leukopenia. They become viremic and shed virus for up to 15 days. Clinical signs include depression, anorexia, oculonasal discharge and decreased milk production. Diarrhea is an inconsistent finding, but when it is present morbidity is generally very high while mortality is very low [4].

Bovine viral diarrhea virus has also been associated with bovine respiratory disease (BRD). Its primary role in this multifactorial disease complex is most likely

immunosuppression [2]. As mentioned earlier, BVDV infected cattle become extremely leukopenic, destroying or inhibiting the normal function of white blood cells [5, 35]. This gives secondary respiratory pathogens such as infectious bovine rhinotracheitis (IBR), bovine respiratory syncitial virus (BRSV), parainfluenza virus type 3 (PI3), *Pasteurella multocida, Manheimian haemolytica,* and *Salmonella spp* a benevolent environment in which to establish infection [2, 3, 36].

In 1993 noncytopathic type II BVDV was isolated from numerous outbreaks of severe acute disease in Canada that killed approximately 25% of the veal calves in Quebec [25, 26]. Clinical signs included pyrexia, pneumonia, diarrhea, and severe hemorrhage throughout the body; thus earning the condition the name hemorrhagic syndrome. At the same time, similar outbreaks were reported in the U.S., primarily in the states surrounding the Great Lakes. Since then this syndrome has been recognized throughout North America with mortality rates in individual herds reaching as high as 20% [2].

Prenatal Infection with BVDV

Bovine viral diarrhea virus infection in immunocompetent pregnant cattle will result in the dam showing the above stated clinical manifestations. However, BVDV readily crosses the placenta and infects the fetus. The result of BVDV fetal infection depends on the stage of gestation in which the fetus is infected.

BVDV infection prior to 50 days gestation

There have been several reports of low conception rates associated with BVDV infection at the time of insemination or in the following 3-4 weeks [37-39]. One reason for this could be that the virus creates a transient endometritis resulting in the inhibition of normal embryonic implantation and development [40]. Acutely infected cows failing to conceive cleared the virus and returned to estrus in a normal interval approximately 20 days later [38].

Another reason for lower conception rates in BVDV infected cattle during the first weeks of gestation is early embryonic death (EED) and reabsorption of the embryo. In one study the rate of EED was significantly greater in infected heifers (44%) as compared to uninfected heifers (9%) [38]. The cow returns to estrus after a prolonged interval and becomes a repeat breeder [2, 38].

These infections often go unnoticed unless there is careful monitoring at the herd level. An increase in the number of services per conception, with a concurrent increase in prolonged, or irregular, estrus cycles is suggestive of early embryonic death; and BVDV should be considered a possible cause.

BVDV infection 50-100 days gestation

Fetal infection 50-100 days in gestation can result in fetal death followed by abortion or mummification. The actual expulsion of the fetus may not occur until several weeks or months following infection [2].

The overall occurrence of BVDV induced abortions is very low (2-7%) [2]. Diagnosing BVDV as the cause of an abortion can be very difficult. Many BVDV

associated aborted fetuses are negative on virus isolation [20, 41]. This may occur because the fetus is often not expelled until several days after death, and in the absence of viable cells, the virus likewise dies [41]. In other cases the fetus may have produced BVDV antibodies which cleared the virus prior to death. The presence of BVDV antibodies in an aborted fetus will confirm intrauterine infection, but does not confirm BVDV as the inciting cause of the abortion [20]. Paired serotesting of the dam may fail to demonstrate seroconversion as the rise in the BVDV antibody titers generally occurs prior to the abortion [4, 20].

BVDV infection 100-150 days gestation

Transplacental infection at approximately 100-150 days in gestation has been associated with various congenital defects, many of which involve the nervous system or eyes [42]. During this period the fetus is in the final stages of organogenesis of the nervous system. The immune system is developed enough that it can generate an inflammatory response to BVDV that can injure the fragile developing nervous tissue. The resultant viral damage includes inhibition of cellular growth, cell differentiation, or cell lysis [2]. Cerebellar hypoplasia is one of the most common defects associated with BVDV infection [2]. Calves with cerebellar hypoplasia have difficulty standing and have a wide base stance with intention tremors. Congenital defects of the eye include varying degrees of blindness, retinal degeneration, and cataracts [2, 42]. Skeletal defects may also be present resulting in small and deformed calves [42].

BVDV infection after 150 days gestation.

After approximately 150 days of gestation, the fetal immune system is fully developed and can mount a response to any infectious agent. Transplacental infection with BVDV during this period usually results in the birth of normal calves seropositive to BVDV [2, 42]. To verify the antibody response occurred in utero, serum must be obtained from the calf prior to the ingestion of colostrum, a potential source of BVDV antibodies for the calf [20]. However, in cases in which the infecting virus is particularly virulent, late term abortions may occur [2].

Persistent infection with BVDV

Persistent infection (PI) with BVDV happens when fetal infection occurs during the period when the immune system is recognizing self-antigens. Virus circulating during this period is recognized as a normal part of the fetus. The fetus becomes immunotolerant to the virus and is unable to clear it from its body, thus becoming persistently infected. The precise time of gestation during which BVDV infection must occur for the establishment of persistent infection is unknown, but PI has rarely been reported after 125 days in gestation [2, 5].

Calves born PI with BVDV may appear clinically normal or they may be small and unthrifty [10, 35]. They tend to be more susceptible to developing infections, most likely due to the immunosuppressive effects of the virus [35]. In a cross-sectional study, PI calves were twice as likely to be culled (either died or were sold due to unthriftiness) from the herd during the first year of life as non-PI herdmates [43].

More importantly, PI cattle serve as the main reservoir for BVDV [3-6, 27]. They continuously shed large amounts of virus into the environment throughout their lives, potentially infecting non-immune herdmates. While a large percentage of PI cattle die within the first year of life, some do survive to adulthood and give birth [43]. Calves born to PI cows will themselves be PI because the virus would have been present during that critical period of immune system development. Thus, a PI cow will always give birth to a PI calf, but a PI calf does not necessarily have a PI dam.

Only noncytopathic strains of BVDV have been isolated from PI cattle [5, 30]. Furthermore, BVDV immunotolerance is strain specific. While PI calves will not form virus neutralizing antibodies to the strain of BVDV they are immunotolerant to; it does not preclude them from becoming infected with a heterologous BVDV strain and developing antibodies to that strain [30]. If however, a PI animal is infected with a cytopathic BVDV strain that is homologous to the noncytopathic strain with which it is infected, it will develop a fatal infection known as mucosal disease [30, 44].

Mucosal Disease

Mucosal disease occurs when a PI animal becomes superinfected with a cytopathic BVDV strain that is homologous to the noncytopathic strain to which it is immunotolerant [5, 44]. This is evidenced by the fact that both noncytopathic and cytopathic BVDV are isolated from cattle with mucosal disease, but these cattle have no viral neutralizing antibodies [44]. The origin of the cytopathic strain may be external, either a cytopathic field strain or a similar modified-live vaccine strain [2]. More likely,

the cytopathic BVDV arises within the PI animal as a mutation of the persistent noncytopathic strain [44].

Clinical signs associated with mucosal disease include pyrexia, depression, anorexia, weight loss, and severe dehydration. Physical examination often reveals oral lesions involving the lips, gingival margins, tongue, and dental pad. These lesions may coalesce and ulcerate, leaving large areas of mucosal necrosis and sloughing. Ptalism and hypersalivation often accompany these oral lesions. Similar ulcerating lesions may also be present on the vulva, teats, and interdigital areas resulting in lameness. Diarrhea may be intermittent, but can become chronic. On postmortem examination areas of necrosis and mucosal sloughing are present throughout the gastrointestinal tract, thus giving rise to the name mucosal disease [2, 30]

The disease is generally acute with death occurring 3-10 days after onset of clinical signs. Occasionally affected cattle will linger for months before dying of severe debilitation or are euthanitized for humane reasons [2, 5, 44]

The occurrence of mucosal disease is sporadic with mortality approaching 100% in affected cattle [44]. However, some herds have reported outbreaks of mucosal disease with up to 20% of the cattle affected. These outbreaks of mucosal disease typically occur in herds where there is an age cohort of PI calves. The virus in one PI calf mutates, and the calf starts shedding a cytopathic virus. This virus then infects its PI pen mates resulting in an epidemic of mucosal disease [44]. There have also been reports of vaccine-associated outbreaks of mucosal disease [2, 44]. Most commercial modified-live BVDV vaccines contain cytopathic virus. Isolation of noncytopathic and cytopathic

BVDV identical to the vaccine strain from cattle dying of mucosal disease 2-4 weeks following vaccination suggests that the vaccine can induce the disease [44].

Prevalence and Incidence of BVDV infection

Evidence of BVDV infection has been found in cattle populations worldwide. The prevalence of BVDV infections is generally expressed either as the percent of seropositive cattle or the percent of PI cattle in a population [6]. BVDV antibodies stimulated by natural infection decrease very slowly over time, and infected animals remain antibody positive for life [30]. Therefore, the prevalence of BVDV antibody carriers reflects the proportion of cattle previously exposed to BVDV, except in areas where cattle are vaccinated.

Table 1.1. Prevalence of BVDV seropositive cattle in various surveys

Country*	Source of Population	No. of Cattle	No. of Herds	Seropositive Cattle (%)	Reference
Croatia	Dairy herds with reproductive problems	NK	4	79.2%	[45]
India	Serum samples negative for rinderpest virus	327	NK	15.29%	[46]
Venezuela	Cattle in Apure State	615	NK	36%	[47]
Slovenia	Whole herds	7968	354	18%	[48]
Poland	Bulls in Artificial Insemination Stations	175	NK	86%	[13]
Northern Italy	unvaccinated dairy herds with reproductive problems	704	29	53.3%	[49]
Belgium	Belgian White-Blue herds	9685	61	65.5%	[14]
England & Wales	Calves and adults	1593	133	62.5%	[50]
UK	NK	18,759	NK	64.9%	[9]
Denmark	Randomly collected sera from 2 slaughterhouses	1332	NK	78%	[51]
Denmark	Whole dairy herds	2570	19	64%	[10]
Sweden	Breeding heifers	711	NK	41%	[7]
Sweden	Adult cows	413	15	45.5%	[52]
Norway	Adult dairy cows	1133	187	18.5%	[53]
United States	Calves and adults	3157	66	89%	[11]
United States	Whole dairy herds with no PI's & no vaccination program	794	5	29%	[12]
United States	Unvaccinated beef cow-calf herds	1755	119	57%	[54]
New Zealand	Beef cattle	140	NK	63%	[55]

NK = Not Known

* BVDV vaccination is prohibited in all countries listed except the United States

The prevalence of BVDV seropositive animals as determined by various surveys is given in Table 1.1. There is a wide range in seroprevalence (15-89%), and a variety of regions and countries represented. The prevalence of PI cattle is given in Table 1.2. In four of the five surveys that are represented in both tables, a PI prevalence of approximately 1% (range 0.75-1.7%) was associated with a BVDV seroprevalence of greater than 60% (64%, 65.5%, 86%, and 89% respectively). It makes intuitive sense that cattle populations with a high BVDV seroprevalence would also have a relatively higher prevalence of PI cattle; because the more cattle exposed, the greater the chances of PI calves being born.

Table 1.2. Prevalence of BVDV PI cattle in various surveys

Country	Source of Population	No. of PI cattle	Total no. of cattle	Percent PI cattle	Reference
UK	Clinically normal calves and cows	4	924	0.4%	[8]
UK	Herds suspected of having BVDV infection	57	3151	1.8%	[9]
Denmark	Representative dairy herd survey	28	2570	1.1%	[10]
Sweden	Breeding heifers	9	711	1.3%	[7]
United States	Calves and cows	54	3157	1.7%	[11]
United States	20 dairy herds in Michigan	71	5481	0.13%	[12]
Poland	Bulls in Artificial Insemination Stations	2	219	0.9%	[13]
Belgium	Belgian White-Blue herds	73	9685	0.75%	[14]
Canada	Feedlot calves	5	5129	0.1%	[15]

Given the high mortality of PI cattle [43], it was determined that the prevalence of PI cattle in a population infected with BVDV at its maximum level will not exceed 2% [1]. This suggests that BVDV infection is approaching its maximum possible level in several of the survey populations in Table 1.2.

The incidence of BVDV infection within herds is difficult to quantify, often because the prevalence is unknown. In a 3-year Danish study new BVDV infections were identified in eight out of nine dairy herds, corresponding to an annual incidence risk of new infections of 52% [56]. In Sweden, serial examinations of bulk tank milk antibody levels are compared. A rise in titers suggests recent BVDV infection. A significant increase in titers was found in five out of 43 herds in one study and seven out of 91 in another. This corresponds to an annual incidence rate of 12% and 8% respectively [16, 57].

Transmission of BVDV infection

The spread of BVDV is most efficient by direct contact between infected and susceptible animals, but infection by indirect means may also occur [6]. Virus has been isolated from virtually every secretion and excretion of infected animals including: blood, nasal discharge, saliva, tears, milk, urine, semen, uterine fluids, and feces [3-6, 30]. Transmission of BVDV can be either vertical or horizontal [6]. Vertical transmission from dam to offspring is extremely efficient; the results of which have previously been discussed as the consequences of prenatal infection.

Horizontal transmission of BVDV occurs by direct or indirect means. Nose-to-nose contact appears to be the most efficient method, with the primary route of infection

being oronasal [6]. Persistently infected cattle are the main reservoir for BVDV as they continuously shed large amounts of virus throughout their lives [3-6, 30]. Transiently infected cattle are also sources of BVDV, although to a much lesser extent than PI cattle. Transiently infected cattle typically shed virus for 2-10 days, and the amount of virus shed is much lower than in PI cattle [6].

Indirect transmission involves an intermediate vehicle that transmits the pathogen between infected and susceptible animals. Indirect transmission of BVDV has been documented to occur through the use of live and contaminated vaccines or health products [1, 58, 59], contaminated needles [6], biting flies [60], and various other fomites including contaminated veterinary equipment, feed, and people [3, 6]. However, BVDV is relatively easily inactivated outside the host, so indirect transmission probably plays only a minor role in the transmission of the virus [6].

Transmission of BVDV has been demonstrated to occur between small ruminants and cattle [61], and BVDV has been isolated from pigs and a wide variety of captive and free-living ruminants [6, 62]. Clinical disease is rarely seen in species other than cattle and sheep. It is unknown if vertical transmission and persistent infection occurs in these animals [62]. In northern Norway a survey detected a higher prevalence of BVDV seropositive reindeer than cattle and sheep [62, 63]. High BVDV antibody titers have also been detected in buffalo and wildebeest with no known contact with cattle [62, 64, 65]. These studies suggest that a wildlife reservoir for BVDV exists, although its importance in transmitting infection to domestic cattle is unknown [62].

Risk Factors for BVDV infection

Houe, et al compared the incidence and prevalence of BVDV infection in Denmark and Michigan and looked at possible risk factors. They found that the prevalence of PI cattle was 10.8 times greater in Denmark than in Michigan. Several factors were associated with the lower prevalence in Michigan including: a lower cattle density, less pasturing of cattle, less purchasing of cattle, and the use of vaccination programs [66].

The higher the cattle density, or larger the herd size, the greater the opportunity for an infected animal to introduce BVDV into the population and allow the virus to propagate. This is especially true in management systems where cattle are kept in strict confinement. Under these conditions, a single PI calf infected over 90% of the herd before it reached 4 months of age [67]. In the Houe, et al study, cattle per square kilometer was 2.5 times greater in Denmark than in Michigan [66]. Further evidence for an increased risk of BVDV infection in areas with high cattle density is seen in Scandinavian countries. In these countries, the seroprevalence of BVDV is significantly higher in the southern regions as compared to the northern regions where the cattle population density is low and herd size smaller [7, 10, 53].

Another practice that dramatically increases the risk of BVDV infection is the commingling of cattle either through sale, show, or common pasturing (a common practice in the Alps) [1, 68]. Any situation which could potentially expose a BVDV naïve animal to an infected animal must be considered a risk factor.

In North America, where BVDV vaccines are available, the lack of, or an inadequate, vaccination program is considered a risk factor for BVDV infection [59, 69].

When done properly, vaccination decreases the susceptibility of the population to infection. To underscore this, few, if any, BVDV outbreaks have occurred in properly vaccinated herds [70].

Diagnosis of BVDV infection

The complex pathogenesis and many clinical manifestations of BVDV infections make it extremely difficult to make a presumptive diagnosis without diagnostic laboratory confirmation. Moreover, an accurate diagnosis of the existence of BVDV within in a herd is imperative for the development of practical and economical herd management strategies. Recent advances in molecular techniques has led to many assays for the detection of BVDV, viral antigens, or antibodies including: PCR, enzyme-linked immunosorbant assay (ELISA), and immunohistochemistry. Each of these newer tests has certain advantages, but virus isolation and virus neutralizing antibody titration remain the gold standards of BVDV diagnosis [20].

BVDV is readily isolated from PI cattle. These cattle are generally viremic throughout their lives [3-6, 30], producing large amounts of virus that can be readily isolated from serum [20]. Transiently infected cattle, however, shed a much smaller volume of virus for a relatively short period of time. Some transiently infected cattle may be positive on virus isolation for only 2-3 days during the course of infection, and few are seldom positive for more than 12 days [20]. The virus is widely disseminated throughout the body, but it has an affinity for lymphoid tissues. Therefore, the mononuclear cells in the buffy coat obtained from whole blood is the best sample for virus isolation, particularly in live cattle. Tissues from the spleen, Peyer's patches of the

small intestine, mesenteric lymph nodes, and thymus are the best samples to submit for virus isolation from aborted fetuses or post-mortem [20].

Serum virus neutralization assays are very sensitive and specific for the detection of BVDV antibodies [20, 71]. The specificity of these assays is dependent on the reference strain of virus used [71]. Unfortunately there is no universally accepted reference strain used by laboratories [20]. It is important to keep this in mind when interpreting results, as the reported titers will depend on how homologous the infecting virus in the animal is to the strain of virus used in the assay [71]. Due to this, it is becoming more common for laboratories to offer both type I and type II BVDV titers for serological analysis. Also, since the results may vary by day and between laboratories, it is recommended that paired samples be tested simultaneously [20].

Another method for detecting BVDV antibodies is an ELISA. It has a quicker turn-around time as compared to virus neutralization [20, 71], and can be performed on milk as well as serum samples [72]. Regardless of the method used, serological determination of BVDV antibody titers can be a useful tool for ascertaining if animals have been exposed to the virus, but it is necessary to be knowledgeable about the age, vaccination status, and past history of the herd from which the animal originated.

Cattle that have been naturally infected with BVDV develop extremely high titers that last for long periods of time [73, 74]. This means that cattle generally have lifelong immunity to the particular strain with which they were infected. It does not, however, prevent them from being infected with a heterologous strain of BVDV; nor does it necessarily confer protection to the fetus if the cow is challenged while pregnant [75].

Vaccination for BVDV is widely practiced in North America [69]. Both inactivated and modified-live virus vaccines are commercially available. Modified live virus vaccines generally induce higher antibody titers that last for a longer period of time than inactivated vaccines [69]. Thus, when evaluating antibody titers it is important to know if the animal was vaccinated, how long ago, and with what type of vaccine.

Colostrum from BVDV seropositive cows contains BVDV antibodies that are absorbed by the calf following ingestion. These passively derived BVDV antibodies have a half-life of approximately 21 days and will persist in the calf's serum for 4-6 months [5, 22, 76]. Therefore, evaluation of BVDV antibody titers in calves less than six months of age is not very informative, unless the sample is collected prior to colostrum intake [2, 5]

PCR and immunohistochemistry have been used on a variety of tissue samples to detect the presence of BVDV RNA and viral antigen respectively. PCR is most commonly used on bulk tank milk samples to determine if the herd is infected with BVDV [21, 77, 78]. The detection of BVDV antigen in skin biopsies by immunohistochemistry is becoming a popular method for identifying PI cattle [79]

In short, diagnosing BVDV infection is a three-step process. First it is necessary to understand the pathogenesis of the virus in order to identify which animals to test and when. Second, one must be knowledgeable about what laboratory tests are available and the best samples to submit for each test. This is especially challenging as technological advances for the detection of BVDV infection are continuously evolving. Third, it is imperative to be able to interpret the results of the laboratory tests, apply them to each

unique herd situation, and develop a strategy for controlling the spread of BVDV to other animals.

Identification of PI cattle and BVDV infected herds

Several methods have been developed to identify PI cattle and BVDV infected herds and are summarized in Table 1.3.

To identify PI cattle in herds believed to have a problem with BVDV, a complete herd screening method is recommended. This is done by detecting virus [20] or viral antigens [79] in infected cattle. Regardless of the method used, all cattle in the herd must be tested including all calves born during the nine months following initiation of BVDV testing [20]. This is a major commitment for the producer in both time and money. As a result, methods to sub-sample the herd to determine if it is infected with BVDV before committing the resources needed for whole herd screening have been proposed.

To date, three strategies have been developed to identify BVDV infected herds. These include determining the level of BVDV antibody in bulk tank milk, identifying BVDV in bulk tank milk, and analyzing BVDV antibody titers in young heifers.

Bulk tank BVDV antibody

Dairy herds infected with BVDV can be accurately identified by analyzing bulk tank milk samples for the presence of BVDV antibodies. This is most commonly done using an indirect ELISA, the results of which are expressed as optical density absorbence values. It has been shown that the calculated absorbence value from the ELISA ran on a bulk tank milk sample is closely correlated to the prevalence of BVDV antibody positive

cows in the lactating herd. Herds with low absorbence values, < 0.20, had few if any (range 0-26.5%) BVDV antibody positive cows. Meanwhile herds with absorbence values > 0.81 had 87-100% BVDV antibody carriers in the lactating herd [57].

Bulk tank ELISA for BVDV antibodies is the foundation for the national BVDV control and eradication programs in many countries including Sweden, Denmark, Finland, and Norway [16-19]. It has also been useful in determining the incidence of BVDV infection within herds. An increase in the absorbence value of bulk tank milk samples taken on a routine basis is suggestive that BVDV infection has been introduced into the lactating herd [19, 57].

The advantages of using bulk tank antibody levels to identify BVDV infected herds include: the bulk tank milk sample is easily obtained, the ELISA is relatively quick, inexpensive, and is closely correlated to the prevalence of BVDV antibody carriers in the lactating herd. The main disadvantage is that it is only a reflection of the herd's exposure to BVDV. It cannot identify an active infection. Since cattle naturally infected with BVDV generally maintain antibodies for life, the bulk tank milk absorbence value may be high in the absence of circulating virus, and remain so for many years until the antibody positive cattle are culled from the herd. Furthermore, the use of this method is not practical in North America due to the widespread use of BVDV vaccines.

Bulk tank PCR

Detection of BVDV RNA in bulk tank milk samples by PCR has also been demonstrated and proposed as a method of identifying infected herds [21, 77]. Unlike the bulk tank ELISA, PCR is able to identify active BVDV infections because it detects viral

RNA. PCR on bulk tank milk samples has been shown to be 14.6 times more sensitive than virus isolation [21] and able to detect one PI cow in a herd of 162 [77].

In terms of sample collection, turn-around-time, and expense, bulk tank PCR is comparable to the ELISA. It has the advantage over virus isolation in that the virus does not need to be replicating for PCR to be positive. Often if a PI cow is present in the lactating herd, there will also be a high prevalence of BVDV antibody carriers. These antibodies may inactivate virus in the bulk tank sample, such that virus isolation will be negative, but the RNA will still be detected by PCR [21]. Although in another study the presence of BVDV antibodies did not impede virus isolation [78].

A disadvantage of using bulk tank PCR for identifying herds infected with BVDV is that it only screens cows whose milk contributed to bulk tank on the day it was sampled. The results cannot be extrapolated to the other cattle in the herd. In most herds the lactating cows make up less than half the herd. Moreover, the majority of PI cattle seldom survive to become part of the lactating herd [43]. Therefore, a BVDV positive PCR bulk tank milk sample is indicative of an infected herd, but a negative sample does not mean there is not BVDV in the herd. There is also some concern that PCR may not detect all naturally occurring BVDV isolates due to sequence variations in the primer regions [78].

Another concern regarding bulk tank PCR is its sensitivity in detecting acutely infected cows. The reported sensitivity of the test has been based on bulk tank milk samples from herds with PI cows in the lactating herd. PI cows shed a larger amount of virus than acutely infected cows. Thus it is presumed that bulk tank PCR would not be as sensitive in identifying herds with acutely infected cows [77].

Serological evaluation of young heifers (spot test)

Analyzing the BVDV antibody titers of a small group of young unvaccinated heifers, or spot testing, has also been shown to accurately identify herds infected with BVDV [22, 23]. The theory behind this strategy is that a high prevalence of seropositive heifers in a herd is indirect evidence that a PI animal is present in the herd [23]. Young heifers essentially serve as sentinels for circulating virus, which is generally the result of a PI herd mate.

In a Danish study the probability of obtaining at least two out of five seropositive animals 6-18 months of age was calculated using a hypergeometric probability function for herds with and without PI cattle. The probability of at least two of five animals being seropositive was 0.977-1 in 10 herds with PI cattle; while in nine herds without PI cattle, the probability of obtaining two seropositive animals ranged from 0-0.048 [22]. A Michigan study determined that the probability of a PI animal being present in a herd was 0.99 if three out of five animals had BVDV antibody titers $\geq$ 128. Accordingly the probability of a PI animal being in the herd was < 0.01 if three of the five animals had titers $\leq$ 64 [23]. In both of these studies, the probability calculations were performed for herds in which all the cattle had been tested for both BVDV and BVDV antibodies prior to the respective study [22, 23]. This technique has yet to be validated in herds with an unknown BVDV status.

The spot test is more laborious and expensive than bulk tank tests, but it is more representative of the entire herd without the cost and trouble of whole herd testing. It accurately predicts the presence or absence of a current infection, and also gives some insight into the infection history of the herd [80]. As long as virus is circulating within

the herd, the animals in the spot test will be seropositive and will remain so for life. However, once the source of virus is removed, the first animals to become seronegative will be 6-month old calves that have never been exposed to the virus and in which passive antibodies have disappeared [22]. Thus, the interpretation of a negative spot test is that the herd has not been infected during the lifetime of the animals tested [80].

Spot tests can result in herd misclassification. A false positive test may occur if the PI animal is removed from the herd shortly before the samples are obtained [22, 23]. Conversely, if a PI animal is extremely young or is a recent introduction into the herd, the virus may not have had time to disseminate throughout the herd, resulting in a false negative spot test [23].

Table 1.3. Advantages and Disadvantages of various BVDV dairy herd screening tests

Herd Screening Test	Advantages	Disadvantages
Whole herd Screening	▪ Most comprehensive way to identify BVDV infected herds ▪ Identifies PI animals so they can be culled	▪ Very expensive ▪ Takes 9-12 months to complete ▪ All cattle in herd must be handled to be bled
Bulk Tank BVDV antibody	▪ Sample easily obtained ▪ Quick and inexpensive ▪ Positively correlated with the prevalence of BVDV antibody carriers in the herd	▪ Cannot identify active infection due to long term antibody carriers ▪ Cannot be used in vaccinated herds ▪ Only reflects antibody status of lactating herd
Bulk Tank PCR	▪ Sample easily obtained ▪ Quick and inexpensive ▪ Identifies active BVDV infection	▪ Only screens lactating herd ▪ Poor sensitivity detecting transiently infected cows ▪ PI's rarely survive to enter lactating herd
Serological Evaluation of Young Heifers (Spot Test)	▪ More representative of entire herd than bulk tank tests ▪ Accurately predicts presence or absence of active infection ▪ Quick ▪ Fairly inexpensive	▪ Sample collection more labor intensive than bulk tank tests ▪ Slightly more expensive than bulk tank tests ▪ Occasional herd misclassification possible

Control and prevention of BVDV

Circulation of BVDV essentially stops once PI cattle are removed from the herd [80]. Therefore, identifying and removing PI cattle is a key component of any BVDV control and prevention program. This is done using various approaches that involve the use of diagnostic tests in target populations within the herd. Other considerations when developing a BVDV control program are herd biosecurity and the use of vaccines.

Once it is verified that BVDV exists within a herd and all PI cattle have been identified and removed, the next step is to prevent the herd from becoming reinfected with BVDV. This requires implementing strict herd biosecurity. Ideally the herd will be kept closed, meaning no outside cattle are brought into the herd. If a closed herd is not an option, then all incoming cattle should be tested to ensure that they are not PI before being introduced into the herd [17]. Given that pregnant cattle can carry PI calves even though they themselves are not PI [4, 27], it is preferable that only nonpregant cattle be purchased. If pregnant cows are purchased, it is important that their calves be isolated as soon as they are born and tested to determine infection status. All PI calves should be removed from the herd immediately [17].

If the farm is unwilling or unable to invest in identifying and removing PI cattle or enforce biosecurity, then there will always be a risk for reinfection by BVDV. Vaccinating for BVDV is an option for controlling the spread of the virus in such herds. There is no set vaccination protocol for controlling BVDV. A vaccination program should be custom designed to each herd based on the existing prevalence of BVDV in the herd, perceived risk of BVDV infection, and overall herd management.

Currently there are over 140 commercial BVDV vaccines licensed for use in the United States [69]. Most of these are multivalent vaccines that contain BVDV and other viral and bacterial agents. Both modified-live virus vaccines and inactivated vaccines are available. Historically, BVDV vaccines contained a single strain of cytopathic type I BVDV [69]. With the emergence and recognition of severe disease caused by type II BVDV, newer vaccines now contain both type I and type II strains [81].

There are advantages and disadvantages in using modified live virus vaccines. Modified live virus vaccines contain attenuated virus such that viral replication and virulence is reduced. The major advantage of modified live virus vaccines is that a single dose will stimulate a rapid immune response that generally will protect the animal for at least 1 year [69, 73]. Other advantages include the stimulation of better cell-mediated immunity and broader antigenic protection [73]. Disadvantages include inactivation of the vaccine and failure of immunization if it is handled improperly, and reversion to virulence resulting in postvaccinal disease. Modified live virus vaccines will also cause transplacental infection and should not be given to pregnant cattle [59, 69].

Inactivated BVDV vaccines likewise have advantages and disadvantages. The main advantage is that they are relatively safe and can be used in pregnant cattle without the fear of fetal infection. Disadvantages are that at least two doses 2-4 weeks apart are needed to achieve primary vaccination, and protection only lasts about 4-6 months [69, 73]

BVDV vaccines are extremely effective in protecting the vaccinated animal from developing clinical disease (90-100% protection in various vaccination-challenge trials), but provide only low to moderate protection for the fetus against transplacental infection

(25-86% protection in vaccination-challenge trials) [59, 75]. Consensus is that inactivated vaccines fail to provide adequate fetal protection. Meanwhile, a study determined that modified live virus vaccine provided a reasonable degree of efficacy against fetal infection (10 of 12 calves protected) for at least three months following vaccination [75].

Control and prevention of BVDV infection is extremely challenging. Ideally all PI cattle are identified and removed from a herd, strict biosecurity measures implemented to prevent reinfection, and a BVDV vaccination program initiated. Even without identification of PI cattle or biosecurity, postnatal BVDV infections can be effectively controlled through the judicious use of vaccines. However, it is questionable if vaccination alone will protect the fetus from transplacental infection.

Conclusion

Worldwide, BVDV is one of the most important and economically damaging viral diseases of cattle. The complex nature of the virus and its many clinical manifestations has made control and prevention of BVDV very challenging and expensive. Developing a plan for controlling BVDV requires knowledge of the pathogenesis of the disease as well as a full understanding of herd history and dynamics. Each herd infected with BVDV is unique, requiring a control program that is customized specifically for the needs and management of that particular herd.

CHAPTER 2

SEROLOGICAL EVALUATION OF FIVE UNVACCINATED HEIFERS TO DETECT HERDS WITH CATTLE PERSISTENTLY INFECTED WITH BOVINE VIRAL DIARRHEA VIRUS

Abstract

Objectives. Validate serological evaluation of five unvaccinated heifers, 6-12 months old, as a method for identifying herds containing cattle persistently infected (PI) with bovine viral diarrhea virus (BVDV).

Sample Population. Fourteen dairy herds with a history of BVDV infection, health problems consistent with BVDV, or at risk for contracting BVDV.

Procedure. Five unvaccinated heifers, 6-12 months old, were randomly selected and neutralizing antibody titers for type I and type II BVDV determined. A herd was classified as likely to contain PI cattle when at least three heifers had antibody titers $\geq$ 128. Virus isolation was performed on all cattle to identify PI animals and definitively classify herds as infected or uninfected. The genotype of isolated viruses was determined by nested multiplex PCR.

Results. Six herds contained PI cattle. Sensitivity and specificity of serological evaluation of five heifers for identifying these herds was 66% and 100% respectively. In herds containing PI cattle, the predominate BVDV titers in sampled heifers corresponded to the genotype of the isolated virus.

Conclusions. Serological evaluation of unvaccinated heifers 6-12 months of age is an accurate method for identifying herds containing BVDV PI cattle. It is imperative that both type I and type II BVDV antibody titers be determined, since herd misclassification may occur if only type I titers are determined. The genotype of BVDV found in PI cattle can be predicted by the predominate virus neutralizing antibody titers found in tested heifers.

Clinical relevance. Serological evaluation of five unvaccinated heifers can be used as a screening tool to determine if a herd is likely to contain cattle persistently infected with BVDV.

Introduction

Diseases caused by bovine viral diarrhea virus (BVDV) result in an estimated annual loss of $10-40 million per million calvings [1, 28]. Due to the complexity of the virus and its many clinical manifestations, BVDV is also one of the most difficult diseases to diagnose and control.

Cattle persistently infected (PI) with BVDV are the major reservoir for viral transmission within and between herds [3-6, 27]. PI cattle are immunotolerant to BVDV and continuously shed large amounts of virus into the environment throughout their lives. A key to eradicating BVDV is the identification and removal of PI cattle from the herd. This requires testing all cattle in the herd for the presence of virus, which is a major expense in both time and money for the producer. Development of a quick, inexpensive, and accurate way to determine if a herd contains PI cattle prior to committing the resources needed for whole herd testing would be useful.

Several strategies have been developed to identify BVDV infected herds. Detection of BVDV antibodies in bulk tank milk can be used to identify dairy herds exposed to BVDV, and is part of the national eradication programs in many European countries [16-19] where BVDV vaccination is prohibited. However, its use in the United States is limited due to widespread use of BVDV vaccines in lactating cows. Thus, it is impossible to determine if BVDV antibodies in bulk tank milk samples are the result of vaccination or natural exposure.

Detection of BVDV RNA in bulk tank milk samples by polymerase chain reaction (PCR) has been demonstrated, but its usefulness as a herd screening test has not been well documented [20, 21]. Its advantage over the bulk tank milk antibody test is that it detects actual virus rather than animals that have been exposed to or vaccinated for BVDV. It has been reported that bulk tank PCR is able to detect one PI animal in a tank sample containing the milk of 162 cows [77]. However, like the bulk tank antibody test, it only screens cattle whose milk contributed to the tank sample tested. The results cannot be extrapolated to the rest of the cattle in the herd, specifically young replacement heifers. Additionally, its usefulness in larger herds is unknown.

Serological evaluation of a small group of young unvaccinated heifers has been proposed as another method for identifying herds with PI animals [22, 23, 77, 82]. The basis for this strategy is a high prevalence of seropositive heifers in a herd is indirect evidence that a PI animal exists in that herd [23]. Young heifers essentially serve as sentinels for detecting circulating virus, which is generally the result of a PI herd mate.

In a Danish study the probability of obtaining at least two out of five BVDV seropositive animals 6-18 months of age was calculated using a hypergeometric probability function for herds with and without PI cattle. The probability of at least two of five animals being seropositive was > 0.977 in 10 herds with PI cattle; while in nine herds without PI cattle, the probability of obtaining two seropositive animals was < 0.048 [22]. The same hypergeometric probability function was applied to the data from 10 dairy herds in Michigan to determine the optimum sample size and neutralizing antibody titer cutoff value combination needed to accurately identify herds with and without PI cattle. The probability of a PI animal being present in a herd was 0.994 if three of five

heifers 9-18 months of age had BVDV antibody titers $\geq$ 1:128. Conversely, if three of five heifers had titers $\leq$ 1:64, the probability of a PI animal in the herd was < 0.01 [23]. In both of these studies, all cattle had been tested for BVDV and BVDV neutralizing antibodies prior to the calculations being performed. This technique has yet to be validated in herds with unknown BVDV status.

The main objective of this study was to validate the serological analysis of five unvaccinated heifers as an accurate method for identifying dairy herds with PI cattle. The genotype of BVDV isolated from PI cattle was determined and compared to the genotype of the predominate BVDV neutralizing antibodies found in the serological analysis of the heifers in the respective herds. Bulk tank PCR was also performed for each study herd, and a questionnaire regarding basic herd management practices was administered.

Materials and Methods

Selection of herds.

Dairy herds in which the current BVDV status was unknown were asked to voluntarily participate in this study. Participating herds were required to milk 200 cows or less and not vaccinate young stock prior to testing. Herds for this study were selectively chosen based on: recent diagnosis of BVDV, were having health problems consistent with BVDV infection, or deemed to be at risk for contracting BVDV (herds that were introducing new cattle into the herd or did not vaccinate).

Sample Size Determination.

Epi Info[a] was used to determine the sample size needed for this study. The assumptions used for this calculation were that the number of herds with PI cattle would equal that of herds without PI cattle. The specificity for herds with a negative serological evaluation was 95%, while the sensitivity for herds with a positive serological evaluation was 90%. The desired confidence level was 95% with 80% power. To meet the above requirements, a minimum of six herds with BVDV PI cattle and six herds without PI cattle were needed, totaling 12 herds.

Sample Collection.

Blood Samples. Blood samples were collected from all cattle in each herd for virus isolation. Serum samples were collected from all cattle $\geq$ 6 months of age, and whole blood samples were collected from calves < 6 months of age. Serum is the easiest and least expensive sample to process, and therefore was the preferred sample for the majority of the cattle. However, colostral antibodies may neutralize free BVDV in the serum of young calves resulting in false negatives on virus isolation. To increase the chance of identifying PI calves < 6 months of age, virus isolation was performed on buffy coats obtained from whole blood samples.

Follow-up blood samples were collected by the herd veterinarian from all animals testing positive on virus isolation. Cattle were classified as PI if they were positive for BVDV on both samples; or if they were positive on the first test, but were removed from

[a] CDC, Atlanta, GA

the herd (culled due to disease consistent with PI or died) before the follow-up sample could be obtained.

The samples were transported to the lab on ice and processed immediately. Serum samples were stored frozen at –80 C. White blood cells were removed from whole blood samples following hypotonic lyses of red blood cells with a 0.83% solution of ammonium chloride in distilled water. The cells were washed once with 10 ml of 0.01 M (pH 7.6) phospate-buffered saline (PBS), then resuspended in 1 ml of eagle's minimum essential medium (EMEM) with 1% L-glutamine and stored at -80 C.

Bulk tank milk sample. A 250 ml bulk tank milk sample was obtained for PCR analysis from each herd on the day of the whole herd blood sample collection. Somatic cells were isolated from the milk sample by centrifuging the sample at 2000 g for 30 minutes at 4 C. The supernate was removed leaving a cell pellet. The cell pellet was resuspended with 2 ml of RNase free water and centrifuged at 2000 g for 30 minutes. The supernate was removed and the process repeated twice. The pellet was then allowed to thoroughly dry and stored at –80 C.

Questionnaire. A questionnaire covering basic herd management practices was administered to either the herd owner or primary herdsman by the principle investigator at the time of the whole herd blood sample collection.

Sample Testing

Virus Isolation. Virus isolation was performed on all samples using an immunoperoxidase monolayer assay (IPMA) similar to that previously described [51]. Briefly, 15 µl of each sample (serum or buffy coat suspension) was inoculated into 96-well microtiter plates containing a monolayer of bovine turbinate (BT) cells in eagle's minimum essential media (EMEM) containing 10% equine serum and 1% L-glutamine. The plates were incubated for three days at 37 C in humidified air containing 5% CO_2. Samples were passed onto new BT monolayers in 96-well plates on the third day. This was accomplished by inoculating the new cells with 15 µl of the supernate from each well. The plates were again incubated for three days, at which time the plates were drained and the cells rinsed with PBS. The cells were then fixed for 10 minutes in 35% acetone in PBS containing 0.02% bovine serum albumin and then allowed to dry at room temperature. The cells were incubated for 30 minutes at room temperature with 100 µl porcine origin polyclonal BVDV antibody[b], diluted 1:100 in binding buffer (PBS with 0.05% Tween and 2.95% NaCl). The polyclonal antibody was removed and the cells washed three times with wash buffer (PBS with 0.05% Tween). Cells were then incubated for another 30 minutes at room temperature with 50 µl of protein G-horseradish peroxidase[c] diluted 1:2000 with binding buffer. Following washing, the cells were incubated in the dark for 1 hour with 100 µl of substrate solution consisting of 3 amino-9 ethylcarbazole (AEC)[d], in 0.05 M sodium acetate buffer (pH 5.0) and 30% hydrogen peroxide. Two positive controls, one BVDV type I and one BVDV type II, and

[b] National Veterinary Services Laboratory, Ames, IA
[c] Zymed Laboratories, Inc., San Francisco, CA
[d] Sigma, St. Louis, MO

two negative controls were included on each plate. A sample was considered positive if distinct red cytoplasmic staining was present in infected cells. Samples were classified as negative if no red cytoplasmic staining was present.

Herds were definitively classified as infected or uninfected with BVDV based on the presence or absence of a PI animal, as determined by IPMA.

Serological analysis. Five unvaccinated heifers, 6-12 months of age from each herd were randomly selected and serum virus neutralizing antibody titers to both type I and type II BVDV determined. Serology was performed by a microtiter virus neutralization procedure [83] using cytopathic Singer strain[b] as the type I BVDV reference strain and cytopathic strain 125[b] as the type II BVDV reference strain. Sera were inactivated in a 56 C water bath for 30 minutes. The virus neutralization test was set up in 96 well microtiter plates using bovine turbinate cells at an initial dilution 15,000 cells per well. Serial two-fold dilutions ranging from 1:4 to 1:4096 were made for each serum sample. The antibody titer was determined as the highest serum dilution where the cytopathic effect of the respective BVDV reference strain was completely inhibited.

A herd was considered to have a positive serological test if three of five heifers had titers $\geq$ 128 for either type I or type II BVDV. Conversely, if three of five heifers had titers $\leq$ 64, the herd was deemed to have a negative serological test.

Viral genotyping. A nested multiplex PCR was used for genotyping of BVDV isolates as previously described [84]. Briefly, RNA was extracted from BVDV positive blood

[b] National Veterinary Services Laboratory, Ames, IA

samples using the RNeasy RNA extraction kit[e]. Reverse transcriptase and first round PCR were combined in a single step using external primers [84] and a commercial kit, Superscript One-Step RT-PCR[f], according to the manufacturer's recommendations. Template RNA (1 μl) was added to a reaction mixture (50 μl total volume) containing 25 μl reaction mix, 1 μl RT/Platinum *Taq* Mix, and 2 μM of each external primer. Reverse transcriptase was carried out at 53 C for 30 minutes followed by denaturation at 94 C for 4 minutes. First round PCR was cycled 40 times at 94 C for 30 seconds, 53 C for 30 seconds, and 72 C for 1 minute with a final extension at 72 C for 10 minutes. Product DNA from this reaction was used in a second round PCR using multiplex primers [84]. Product DNA (1 μl) was added to a reaction mixture (50 μl total volume) containing 10x PCR buffer[e], 200 μM dNTP's[e], 2.5 units *Taq* DNA polymerase[e] and 4 μM of each primer. Second round PCR consisted of denaturation at 94 C for 4 minutes followed by 40 cycles of 94 C for 30 seconds, 53 C for 30 seconds, 72 C for 1 minute, and a final extension at 72 C for 10 minutes. Products were electrophoresed on a 2% agarose gel and stained with ethidium bromide. Amplified bands at 360 bp corresponded with type I BVDV isolates, while those at 640 bp corresponded to type II BVDV.

Bulk tank PCR. PCR was performed on somatic cells isolated from bulk tank milk samples using the nested multiplex PCR procedure described above.

[e] Qiagen, Valencia, CA
[f] Gibco BRL/Invitrogen Corporation, Carlsbad, CA

Data Analysis.

The results were tabulated in a 2 x 2 contingency table according to the above definitions of BVDV herd classification and positive and negative serological test. The herd sensitivity, herd specificity, and kappa test statistic with accompanying 95% confidence intervals was determined. Herd sensitivity was calculated as the number of herds with PI animals and a positive serological test divided by the total number of herds with PI animals. Herd specificity was calculated as the number of herds without PI animals and a negative serological test divided by the total number of herds without PI animals. The kappa test statistic and respective 95% confidence intervals for sensitivity, specificity and kappa were calculated using Episcope[g]. The same methods were used to compare bulk tank PCR results with herd virus isolation.

Results

Fourteen dairy herds were tested in this study. Most of the herds were open herds, or had purchased cattle within the last five years. Table 2.1 compares herd management practices between herds with PI cattle and herds without PI cattle.

PI animals were identified in six herds resulting in a herd prevalence for this study of 43%. The number and prevalence of PI animals in each herd is summarized in Table 2.2.

[g] University of Edinburgh, Edinburgh, UK

Table 2.1. Descriptive comparison of herd management practices in study herds

	Herds without PI cattle	**Herds with PI cattle**
Open herd	7	6
Closed herd	1	0
Source of cattle		
▪ private herd	5	1
▪ stockyards/auction	2	5
Screened for BVDV	0	1
Segregated before intro. to herd	1	1
Vaccination—Cows		
▪ MLV	1	0
▪ killed 1x/yr	2	1
▪ killed 2x/yr	1	4
▪ killed 3x/yr	2	0
▪ none	2	1
Vaccination—Calves/heifers		
▪ MLV	4	1
▪ killed	1	3
▪ none	3	2
Breeding		
▪ AI only	0	1
▪ Natural Service only	1	3
▪ Combo AI & Natural Service	5	2
Evidence of BVDV*	3	5

* Evidence of BVDV included herds with reproductive problems, herds in which cattle did not respond as expected to treatment, or herds with recent diagnostic results indicating the presence of BVDV.

Table 2.2. Prevalence of PI cattle in study herds

Herd number	BVDV PI in herd	Number of cattle	Number of PI cattle	Percent PI cattle
1	Yes	236	5	2.1
2	Yes	198	1	0.5
3	Yes	379	1	0.26
4	Yes	261	6	2.3
5	Yes	246	3	1.2
6	Yes	355	6	1.7
7	No	123	0	0
8	No	295	0	0
9	No	149	0	0
10	No	109	0	0
11	No	142	0	0
12	No	449	0	0
13	No	113	0	0
14	No	413	0	0
Study totals		3468	22	0.6

Serological test of five random unvaccinated heifers. The results comparing the serological test of five heifers to herd virus isolation are given in Table 2.3.

Table 2.3. Serological test of 5 heifers compared to whole herd virus isolation as a method of preliminary screening dairy herds for cattle persistently infected with BVDV.

	BVDV herd virus isolation		Totals
	Positive	Negative	
Positive Serological Test *	4	0	4
Negative Serological Test **	2	8	10
Totals	6	8	14

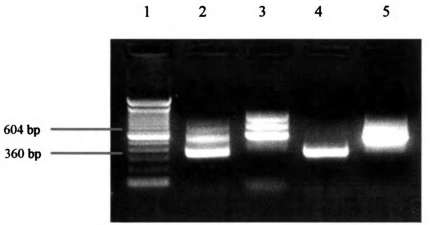
* Positive serological test: herd in which at least 3 of 5 randomly selected, unvaccinated heifers 6-12 mos. of age had BVDV titers $\geq$ 128

** Negative serological test: herd in which at least 3 of 5 randomly selected, unvaccinated heifers 6-12 mos. of age had BVDV titers $\leq$ 64

The herd sensitivity for the serological test was 66% (95% CI 29-100) and herd specificity was 100%. The kappa test statistic for agreement between the two tests was 0.7 (95% CI 0.20-1.20).

Correlation of genotype of virus isolated to predominate BVDV titer in herd. In all herds in which PI cattle were identified, the genotype of the virus isolated corresponded to the predominate BVDV neutralizing antibody titers in the heifers sampled. For example, if the heifers sampled had higher type II BVDV titers than type I, a type II virus was isolated from the herd, and vice versa. Figure 2.1 shows an example of PCR results with type I and type II BVDV isolates.

Figure 2.1. BVDV viral genotyping by nested RT-PCR



Lane 1: 1 kb DNA ladder
Lane 2 & 4: Type I BVDV isolates with amplification bands at 360 bp
Lane 3 & 5: Type II BVDV isolates with amplification bands at 604 bp

Bulk tank PCR. The bulk tank PCR results compared to the whole herd virus isolation are given in Table 2.4.

Table 2.4. Bulk tank PCR compared to whole herd virus isolation as a method of preliminary screening dairy herds for cattle persistently infected with BVDV.

		BVDV herd virus isolation		Totals
		Positive	Negative	
Bulk Tank PCR	Positive	2	0	2
	Negative	4	8	12
Totals		6	8	14

The herd sensitivity of the bulk tank PCR for identifying dairy herds with PI cattle was 33% (95% CI 0-71) and the herd specificity was 100%. The kappa value was a low 0.36 (95% CI 0.04-0.77).

Discussion

The results of this study indicate that serological evaluation of five unvaccinated heifers is an accurate method for determining if a BVDV PI animal is present within a herd. Defining the cutoff for a positive serological test as a herd in which three of five heifers have BVDV neutralizing antibody titers $\geq$ 128 resulted in a herd test with 66% sensitivity, 100% specificity. This means that given a positive serological test, a PI animal could be found within the herd. A negative serological test did not guarantee that a PI animal was not present in the herd, as evidenced by two false negative test results. These two herds (herds 3 and 4 from table 2.2) represented possible scenarios that must be considered when interpreting serological tests. In herd 3 a single PI heifer was identified. At the time of testing she was three months of age and may not have been

shedding virus due to colostral antibody inactivation of free virus. Also, since she had had no contact with the target population, or population of animals from which the serological test samples were taken; those animals had not been exposed to the virus at the time of testing. In herd 4, the six PI heifers identified made up an age cohort within the target population that totaled 13 animals. By chance, three of those PI heifers were selected for the serological test and all had BVDV titers < 4, resulting in a negative serological herd test. The other two heifers sampled from this herd each had titers > 4096. One PI animal was also included in the serological evaluation for herd 1. However, the rest of the heifers in the sample all had extremely high titers such that the herd was correctly classified. Thus, the inclusion of PI animals in the herd sample may influence the results of the serological test, depending on how many PI animals are tested and what cutoffs are used.

The kappa test statistic was 0.7, meaning there was substantial agreement between the serological evaluation of five heifers and whole herd virus isolation, the method used to definitively classify which herds contained PI animals. The kappa statistic does not identify which test is correct, only the level of agreement between them [85]. However, if whole herd virus isolation was the "gold standard" by which herds were identified as having PI animals, then the kappa statistic is an indirect measure of how well the serological evaluation of five heifers identifies herds with PI animals.

The definition of a positive serological test for this study was based on a previous study [23]. Changing the cutoff values used to define a positive serological test would alter the herd sensitivity and specificity of the test. Generally, increasing the titer cutoff or the number of heifers tested with titers greater than a given cutoff improved specificity

while sensitivity was decreased and vice versa. Selecting a cutoff is a subjective process and depends on the relative cost of false negative and false positive test results. For example, in this study the cost of a false positive test result was considered much greater than the cost of a false negative result because a false positive result could lead to extensive testing to identify a PI animal within a herd that did not exist. Therefore, if a different cutoff for a positive serological test were chosen, a greater value would be placed on specificity, even though it would likely mean sacrificing sensitivity.

The overall sensitivity of using serological evaluation of five unvaccinated heifers as a method to screen dairy herds for PI cattle could be improved by retesting those herds that test negative at regular intervals. Had the herds testing negative in this study been retested in six months, it is likely that the two false negative herds would have tested positive. In herd 3, the PI heifer would have been within the target population, and it is likely her pen mates would have been seropositive. The age cohort of PI cattle in herd 4 would no longer be in the target population, and it is probable that the heifers following them would have seroconverted.

Another way that the overall sensitivity of screening a sample of five heifers for BVDV could be improved is to run virus isolation and serum virus neutralization on the heifers in parallel. A positive herd test would be defined as a positive serological test or an animal testing positive on virus isolation. In this study, parallel testing would have resulted in herd 4 testing positive, and the overall sensitivity would have improved from 66% to 83%. Herd 3, however would still have been classified as negative. Either of the above methods to improve sensitivity will increase the overall cost of the herd screening,

but it is minimal when compared to the time and money needed for individual testing of all animals in the herd.

In previous studies [22, 23, 82], herd size did not impact the results of the serological test. Therefore, it is likely that serological evaluation of five unvaccinated heifers will be effective for herds regardless of size, as long as the herd is managed as one unit with the usual cross traffic. Serological evaluation of five unvaccinated heifers will be less likely to be effective if the heifers are housed at some distance from the main farm, or are contracted to a heifer raiser where they are commingled with animals from other herds. In that instance the serological test would reflect the status of the heifer raiser's herd rather than the herd of origin.

A factor that does play a significant role when using serological evaluation of five unvaccinated heifers to identify herds with PI cattle is that both type I and type II BVDV neutralizing antibody titers must be determined. Some labs may only run type I BVDV titers unless both type I and type II titers are specifically requested. In this study, had only type I titers been done, all four of the herds that had a positive serological test would have been negative and misclassified.

There was excellent correlation between the genotype of the virus isolated from a herd and the predominate BVDV neutralizing antibody titers found in the five heifers that were tested from that respective herd. Thus, if the type II titers were higher than the type I titers on the serological test, a type II virus was isolated from the herd. Knowing the genotype of the virus infecting the herd may be useful when designing a vaccination program for that herd. Many BVDV vaccines contain only type I strains, although there are newer vaccines that contain both type I and type II strains. There is some evidence

that type I vaccines will cross-protect against some type II strains [70, 86, 87], but this protection may be incomplete [88], especially against fetal infection. In these herds, it may be wise to choose a vaccine that specifically contains a type II BVDV strain.

For this study, the target population for sampling was heifers 6-12 months of age. Passively derived antibodies are generally undetectable by six months of age and will no longer interfere with the test [22]. As the first animals to become seronegative following the removal of all PI animals from a herd are unvaccinated heifers just older than six months, it has been suggested that the most effective age group to sample is those heifers 6-12 months of age [22]. Also, 12 months is a common age at which producers begin to prepare heifers for breeding and begin vaccinating them for BVDV.

Another method that has been suggested for screening dairy herds for the presence of BVDV PI animals is a bulk tank PCR test. This test was performed on all herds in this study and the results given in Table 2.4. When compared to whole herd virus isolation, the bulk tank PCR sensitivity for identifying herds with PI animals was only 33%, but its specificity was 100%. A low kappa value (0.36), suggested that there was very little agreement between the bulk tank PCR test and the "gold standard" whole herd virus isolation. Part of the reason why the bulk tank PCR test had such poor sensitivity was adult PI cows were found in only three of the six herds with PI animals. Most PI heifers do not survive to make it into the lactating herd. In one study PI calves were twice as likely to be culled (either died or were sold due to unthriftiness) from the herd during the first year of life as non-PI herdmates [43]. Bulk tank PCR was negative for one of the herds that had an adult PI cow. Since it is not known if that PI cow's milk contributed to the bulk tank the day the sample was collected, it is not possible to

determine if the PCR was truly a false negative test (BVDV present in the sample, but not detected by PCR). Regardless, when compared to the serological evaluation of five unvaccinated heifers 6-12 months of age, bulk tank PCR was not an effective method for identifying dairy herds with PI animals.

The data listed in Table 2.1 is of an observational nature only. No statistical analysis or relevance can be applied to it. There was only one completely closed herd in the study. The rest of the herds purchased cattle on a regular basis or had added cattle within the last five years. Of particular note was the source of these purchased cattle. Five of the six herds with PI animals had purchased cattle from the stockyards or auction barn compared to only two of the herds without PI cattle. That does not mean that cattle purchased from the stockyards were the source of BVDV infection for these herds, but one would certainly expect that the stockyard would be good place for herds to acquire BVDV infection due to the stress and commingling of cattle from several sources. Many producers use the stockyards as the market for their unthrifty or sick cattle, some of which could be PI animals. Moreover, this observation is in agreement with that of a previous study looking at risk factors for BVDV infection [66].

The results from this study cannot be extrapolated to the general population of dairy herds, because the herds in this study were not randomly selected. The prevalence of PI cattle within BVDV infected herds ranged from 0.26-2.3%. This corresponds to the prevalence of PI cattle cited in several other studies [7-15], suggesting that the herds in this study are typical of BVDV infected herds. A previous study in Michigan, found PI cattle in 15% of randomly selected dairy herds [12]. The prevalence of PI herds in this study was 43%. These study herds were selectively chosen so that the herd prevalence

would be higher in the study population than the general population. However, for all practical purposes, the herds wanting to screen for BVDV are most likely those that believe they have PI cattle. Therefore, this study population may very well be an accurate reflection of the population in which the serological evaluation of five unvaccinated heifers 6-12 months of age would most likely be applied.

In conclusion, the serological evaluation of five unvaccinated heifers 6-12 months of age is an accurate method to screen herds for BVDV PI animals. The sensitivity of this method can be improved by retesting negative herds at regular intervals or doing virus isolation in parallel with virus neutralization on the five heifers tested. It is important that both type I and type II BVDV neutralizing antibody titers be determined as herds may be misclassified when only type I titers are determined.

CHAPTER 3

GENERAL DISCUSSION

General Discussion

Serological evaluation of five unvaccinated heifers 6-12 months of age, or herd serological test, was determined to be an accurate method for identifying dairy herds with BVDV PI animals. Also, excellent correlation was shown to exist between the genotype of BVDV isolated from PI cattle and the predominate BVDV neutralizing antibody titers of the tested heifers from their respective herds. Knowing if a herd is likely infected with BVDV as well as which genotype of the virus is present is valuable information when developing a BVDV control and vaccination program for that herd. It allows the producer and veterinarian to make informed decisions as to the need for further testing for BVDV in the herd, what type of vaccine should be used, and how often the herd should be vaccinated to control and prevent BVDV infections.

The following is a discussion about the study overall: the issues that arose during the study, how the study could be improved, and issues that need further investigation.

Issues that arose during the study

Overall, the use of serological evaluation of five unvaccinated heifers 6-12 months of age appears to be an accurate method for screening dairy herds for PI cattle. In this study, it was better than bulk tank PCR analysis for BVDV in predicting the presence of PI cattle in a herd, especially in terms of herd sensitivity. This is because it is a better representation of the entire herd. Bulk tank PCR only screens the lactating herd for BVDV, and its results cannot be extrapolated to rest of the herd. Moreover, while it detects cows shedding BVDV, and thus most likely PI animals, the prevalence of PI animals in the lactating herd is generally much lower compared to the rest of the herd,

specifically replacement heifers. This is because few PI heifers survive to become lactating cows [43]. With serological evaluation of heifers, the goal is not to identify active infection, but rather exposure to the virus. In BVDV infected herds, the number of seropositive animals will be far greater than the number of PI cattle. Therefore the herd sensitivity and herd specificity using serological evaluation of five unvaccinated heifers will be higher than that for bulk tank PCR because the prevalence of the condition being sought, BVDV exposure, is greater. Additionally, the heifers sampled for the herd serological test act as sentinels to detect BVDV in their environment. Generally, if the target population has been exposed to BVDV, it is likely the rest of the herd has been exposed as well. Thus serological evaluation of five unvaccinated heifers is a better herd screening test than bulk tank PCR because the prevalence of what is being tested, BVDV exposure versus infection, is higher; and it is a better reflection of the entire herd.

A major concern that arose during this study was how changing the criteria defining a positive herd serological test would affect the herd sensitivity (Se), herd specificity (Sp), and kappa of the test. To investigate, those parameters were calculated for all possible combinations of BVDV neutralizing antibody titer cutoffs and herd sample cutoffs, or the number of heifers tested with titers at or above a given cutoff. These results are summarized in tables 3.1-3.5.

Table 3.1. Herd sensitivity, specificity and kappa values comparing serological evaluation of 5 unvaccinated heifers to whole herd BVD virus isolation when at least 1 of 5 heifers have BVDV antibody titers ≥ cutoff

Cutoff	Se (%)	Se 95% CI		Sp (%)	Sp 95% CI		Kappa	Kappa 95% CI	
		lower	upper		lower	upper		lower	upper
<4	100	100	100	0	0	0	NC	NC	NC
4	100	100	100	75	45	100	0.72	0.22	1.22
8	100	100	100	75	45	100	0.72	0.22	1.22
16	100	100	100	75	45	100	0.72	0.22	1.22
32	83	53.5	100	75	45	100	0.57	0.05	1.09
64	83	53.5	100	75	45	100	0.57	0.05	1.09
128	83	53.5	100	75	45	100	0.57	0.05	1.09
256	83	53.5	100	75	45	100	0.57	0.05	1.09
512	83	53.5	100	87.5	65	100	0.71	0.19	1.23
1024	83	53.5	100	87.5	65	100	0.71	0.19	1.26
2048	83	53.5	100	100	100	100	0.85	0.33	1.37
4096	83	53.5	100	100	100	100	0.70	0.20	1.20

NC = Not calculated

Table 3.2. Herd sensitivity, specificity and kappa values comparing serological evaluation of 5 unvaccinated heifers to whole herd BVD virus isolation when at least 2 of 5 heifers have BVDV antibody titers ≥ cutoff

Cutoff	Se (%)	Se 95% CI		Sp (%)	Sp 95% CI		Kappa	Kappa 95% CI	
		lower	upper		lower	upper		lower	upper
<4	100	100	100	0	0	0	NC	NC	NC
4	83	53.5	100	75	45	100	0.57	0.05	1.09
8	83	53.5	100	75	45	100	0.57	0.05	1.09
16	83	53.5	100	75	45	100	0.57	0.05	1.09
32	83	53.5	100	75	45	100	0.57	0.05	1.09
64	83	53.5	100	75	45	100	0.57	0.05	1.09
128	83	53.5	100	75	45	100	0.57	0.05	1.09
256	83	53.5	100	75	45	100	0.57	0.05	1.09
512	83	53.5	100	100	100	100	0.85	0.33	1.37
1024	83	53.5	100	100	100	100	0.85	0.33	1.37
2048	83	53.5	100	100	100	100	0.85	0.33	1.37
4096	66	29	100	100	100	100	0.70	0.20	1.20

NC = Not calculated

Table 3.3. Herd sensitivity, specificity and kappa values comparing serological evaluation of 5 unvaccinated heifers to whole herd BVD virus isolation when at least 3 of 5 heifers have BVDV antibody titers ≥ cutoff

Cutoff	Se (%)	Se 95% CI		Sp (%)	Sp 95% CI		Kappa	Kappa 95% CI	
		lower	upper		lower	upper		lower	upper
<4	100	100	100	0	0	0	NC	NC	NC
4	66	29	100	75	45	100	0.42	-0.11	0.94
8	66	29	100	87.5	65	100	0.55	0.04	1.07
16	66	29	100	87.5	65	100	0.55	0.04	1.07
32	66	29	100	100	100	100	0.70	0.20	1.20
64	66	29	100	100	100	100	0.70	0.20	1.20
128	66	29	100	100	100	100	0.70	0.20	1.20
256	50	10	90	100	100	100	0.53	0.07	1.00
512	50	10	90	100	100	100	0.53	0.07	1.00
1024	50	10	90	100	100	100	0.53	0.07	1.00
2048	50	10	90	100	100	100	0.53	0.07	1.00
4096	33	0	71	100	100	100	0.36	-0.04	0.77

NC = Not calculated

Table 3.4. Herd sensitivity, specificity and kappa values comparing serological evaluation of 5 unvaccinated heifers to whole herd BVD virus isolation when at least 4 of 5 heifers have BVDV antibody titers ≥ cutoff

Cutoff	Se (%)	Se 95% CI		Sp (%)	Sp 95% CI		Kappa	Kappa 95% CI	
		lower	upper		lower	upper		lower	upper
<4	100	100	100	0	0	0	NC	NC	NC
4	50	10	90	100	100	100	0.53	0.07	1.00
8	50	10	90	100	100	100	0.53	0.07	1.00
16	50	10	90	100	100	100	0.53	0.07	1.00
32	50	10	90	100	100	100	0.53	0.07	1.00
64	50	10	90	100	100	100	0.53	0.07	1.00
128	50	10	90	100	100	100	0.53	0.07	1.00
256	50	10	90	100	100	100	0.53	0.07	1.00
512	50	10	90	100	100	100	0.53	0.07	1.00
1024	50	10	90	100	100	100	0.53	0.07	1.00
2048	33	0	71	100	100	100	0.36	-0.04	0.77
4096	33	0	71	100	100	100	0.36	-0.04	0.77

NC = Not calculated

Table 3.5. Herd sensitivity, specificity and kappa values comparing serological evaluation of 5 unvaccinated heifers to whole herd BVD virus isolation when all 5 heifers have BVDV antibody titers ≥ cutoff

Cutoff	Se (%)	Se 95% CI		Sp (%)	Sp 95% CI		Kappa	Kappa 95% CI	
		lower	upper		lower	upper		lower	upper
<4	100	100	100	0	0	0	NC	NC	NC
4	33	0	71	100	100	100	0.36	-0.04	0.77
8	33	0	71	100	100	100	0.36	-0.04	0.77
16	33	0	71	100	100	100	0.36	-0.04	0.77
32	33	0	71	100	100	100	0.36	-0.04	0.77
64	33	0	71	100	100	100	0.36	-0.04	0.77
128	33	0	71	100	100	100	0.36	-0.04	0.77
256	33	0	71	100	100	100	0.36	-0.04	0.77
512	33	0	71	100	100	100	0.36	-0.04	0.77
1024	33	0	71	100	100	100	0.36	-0.04	0.77
2048	33	0	71	100	100	100	0.36	-0.04	0.77
4096	17	0	46	100	100	100	0.19	-0.12	0.49

NC = Not calculated

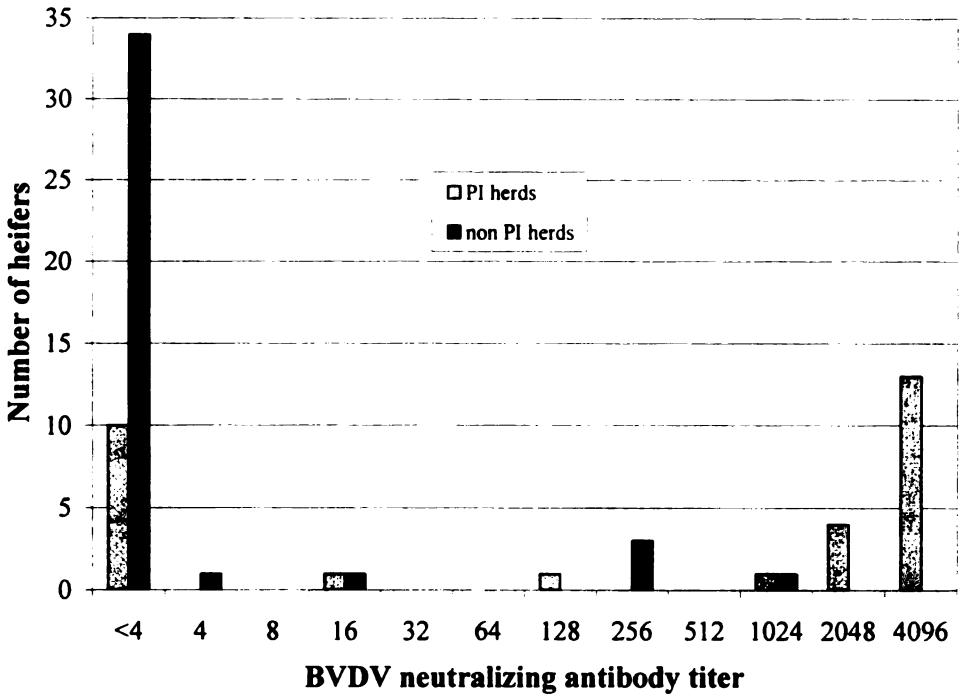
Changing either the BVDV neutralizing antibody titer or herd sample cutoff values that define a positive serological test will affect the sensitivity, specificity, and kappa value of the test as seen in tables 3.1-3.5. The reason the three of five heifers ≥ 128 titer cutoff was chosen for this study was due to findings in a previous study [23]. The ideal cutoff for a herd test would be one that maximizes sensitivity and specificity. Other things that must be considered when choosing a cutoff is the distribution of test results between herds with and without PI cattle, the prevalence of seropositive animals in the target population, and the consequence of false negative and false positive herd test results.

Tables 3.1-3.5 summarize the herd serological test results when the cutoff for the herd sample cutoff is kept constant while the titer cutoff is changed. In each table as the titer cutoff increases, sensitivity of the test decreases, while specificity of the test

increases. Likewise, as the herd sample cutoff is increased for a given titer cutoff, sensitivity tends to decrease while specificity increases. In general, as the test cutoff is raised, either at the herd level (herd sample cutoff) or the individual animal level (titer cutoff), specificity will improve while sensitivity will get worse.

The prevalence of BVDV seropositive animals in the target population will not affect the sensitivity and specificity of the individual animal serum virus neutralization test, but it will affect the sensitivity and specificity of the serological test at the herd level. In this study the prevalence of BVDV seropositive heifers in herds with PI animals was 75% while that in herds without PI animals was only 15% (table 3.6). Thus, it is much more likely the serological test will be positive in herds with PI animals, regardless of the test cutoff, because there are more seropositive animals in the target population when compared to herds without PI animals. In effect, as the prevalence of seropositive animals in the target population increases, sensitivity will also increase while specificity decreases.

Table 3.6. Distribution of BVDV neutralizing antibody titers in sampled heifers.



The final thing to consider when selecting a cutoff for a test is the relative cost of false positive and false negative results. This is a subjective measure and may change given different scenarios. For this study the cost of a false positive test result was considered much greater than the cost of a false negative result, because a false positive result could lead to extensive testing to identify nonexistent PI animals. Given the inverse relationship between sensitivity and specificity, a greater value would be placed on specificity when choosing the best cutoff value for the serological test in this instance. If serological evaluation of five unvaccinated heifers was used as part of a national eradication program, one would want to maximize sensitivity to ensure that all herds with

PI cattle are identified. This would be done at the risk of increased false positive test results.

Going back to tables 3.1-3.5, it can be argued that the best definition for a positive serological test was not three of five heifers with titers $\geq$ 128. In table 3.2, where the herd sample cutoff was two of five heifers, the test results across antibody titer cutoff values of 512, 1024, and 2048 are the same. However, when compared to the three of five heifers with titers $\geq$ 128 cutoff, the herd sensitivity is improved from 66% to 83%. The kappa test statistic is also better for these cutoffs (0.85). The difference between the two cutoffs is due to the misclassification of herd 4 at the three of five level, but not the two of five level. Therefore, for this study population, a better definition for a positive serological test would have been two of five heifers with BVDV titers $\geq$ 512. Had a larger number of herds been tested, it is likely that the optimum cutoff would again change.

The same argument used for the two of five heifers with titers $\geq$ 512 cutoff could be made for using a one of five heifers with a BVDV titer $\geq$ 2048 (table 3.1) cutoff. Its test values are the same as those found for the optimum cutoff in table 3.2. However, when choosing an extremely low herd sample cutoff such as one of five, specificity is relatively low except at extremely high titer cutoffs ($\geq$ 2048). Therefore, it may not be the best cutoff to choose if specificity is more important than sensitivity. Accordingly, if an extreme titer cutoff ($\geq$ 1024) is chosen, sensitivity is likely to be affected, especially at high herd cutoff values (tables 3.4 & 3.5). Thus, when choosing a cutoff where the values remain constant over several variables, as in this study, it might be best to choose the more moderate cutoff to preserve both sensitivity and specificity.

How the study could be improved

Part of the variation seen in herd sensitivity and herd specificity across various cutoffs in this study was due to the small study population (n=14). Moreover, two of the BVDV infected herds represented rare scenarios resulting in their misclassification by serological testing at various cutoff levels (data for all study herds is given in table 3.7). With a small study population the magnitude of effect a false negative or false positive test has on sensitivity and specificity is much greater than in a larger population. A good example of this occurred with herd 4 (table 3.7), in which there was an age cohort of six PI heifers in a target population of 13. Three PI heifers were randomly selected for the serological evaluation. As a result, the herd was classified as negative when the definition for a positive serological test was three of five heifers with titers $\geq$ 128, and the sensitivity of the test was 66% (table 3.3). However, that herd was correctly classified when the cutoff for a positive serological test was two of five heifers with titers $\geq$ 512, and the corresponding sensitivity of the test became 83% (table 3.2). Thus, there was a 17% difference in the test's sensitivity given the misclassification of one herd as a false negative in a study population of only 14. Contrast this to the sensitivity of the test had there been 25 BVDV infected herds in this study, still with two herds being false negatives on herd serological test. The sensitivity for the herd serological test under these circumstances would then be 92%. Adjusting the cutoff so that only one herd was a false negative would yield a sensitivity of 96%, only a 4% increase in sensitivity compared to the 17% increase seen previously. The specificity for this study remained fairly constant because there were no false positive herds, but the above principles would undoubtedly be the same.

Table 3.7. PI cattle in herd, BVDV titers in the 5 sampled heifers and their BVDV status, the genotype of BVDV isolated from infected herds, and bulk tank PCR results for all herds in the study

Herd	PI cattle in herd	Heifer No.	BVDV antibody titers		VI status	Genotype isolated	Bulk tank PCR
			Type I	Type II			
1	Yes	1	<4	<4	+	Type II	Negative
		2	<4	>4096	-		
		3	16	>4096	-		
		4	4	2048	-		
		5	4	1024	-		
2	Yes	1	16	>4096	-	Type II	Positive
		2	128	>4096	-		
		3	32	>4096	-		
		4	64	>4096	-		
		5	4	2048	-		
3	Yes	1	16	4	-	Type I	Negative
		2	<4	<4	-		
		3	<4	<4	-		
		4	<4	<4	-		
		5	<4	<4	-		
4	Yes	1	>4096	1024	-	Type I	Negative
		2	<4	<4	+		
		3	<4	<4	+		
		4	>4096	512	-		
		5	<4	<4	+		
5	Yes	1	8	2048	-	Type II	Positive
		2	<4	<4	-		
		3	<4	<4	-		
		4	512	2048	-		
		5	64	128	-		
6	Yes	1	1024	>4096	-	Type II	Negative
		2	128	>4096	-		
		3	1024	>4096	-		
		4	64	>4096	-		
		5	64	>4096	-		
7	No	1	<4	<4	-	Not Isolated	Negative
		2	<4	<4	-		
		3	<4	<4	-		
		4	<4	<4	-		
		5	<4	<4	-		

Table 3.7 continued

Herd	PI cattle In herd	Heifer No.	BVDV antibody titers		VI status	Genotype isolated	Bulk tank PCR
			Type I	Type II			
8	No	1	<4	<4	-	Not Isolated	Negative
		2	<4	<4	-		
		3	<4	<4	-		
		4	<4	<4	-		
		5	<4	<4	-		
9	No	1	256	128	-	Not Isolated	Negative
		2	16	<4	-		
		3	1024	128	-		
		4	<4	<4	-		
		5	<4	<4	-		
10	No	1	<4	<4	-	Not Isolated	Negative
		2	<4	<4	-		
		3	<4	<4	-		
		4	<4	<4	-		
		5	<4	<4	-		
11	No	1	<4	<4	-	Not Isolated	Negative
		2	<4	<4	-		
		3	<4	<4	-		
		4	<4	<4	-		
		5	<4	<4	-		
12	No	1	<4	<4	-	Not Isolated	Negative
		2	<4	<4	-		
		3	<4	<4	-		
		4	<4	<4	-		
		5	<4	<4	-		
13	No	1	<4	<4	-	Not Isolated	Negative
		2	<4	<4	-		
		3	<4	<4	-		
		4	<4	<4	-		
		5	<4	<4	-		
14	No	1	<4	<4	-	Not Isolated	Negative
		2	<4	<4	-		
		3	256	8	-		
		4	4	<4	-		
		5	256	16	-		

Therefore, had a larger study population been used, it is likely the amount of variation in both sensitivity and specificity between cutoff values would have been minimized.

As mentioned, in this study there were two herds with PI cattle that tested negative at the reference cutoff of three of five heifers with titers $\geq$ 128. The reasons for the false negative test results were: including PI cattle in the serological test, or, as in herd 3 (table 3.7), having an extremely young PI animal that either was not shedding virus or had not yet infected the target population. False positive test results, although not represented in this study, are possible. A false positive serological evaluation could result if all PI animals had recently been removed from the herd, and not enough time had elapsed for the target population to become seronegative. Another scenario in which a false positive serological evaluation could be obtained is if the heifers sampled were raised at a different facility and commingled with cattle from other herds such as at a contracted heifer grower facility. In that situation, the serological evaluation would reflect the BVDV status of the herd in which the heifers are being raised rather than the herd of origin.

Theoretically, the sensitivity for the serological evaluation of five unvaccinated heifers 6-12 months of age could be improved by either doing virus isolation in parallel with serum virus neutralization on the sampled heifers or retesting the herd at regular intervals. Had virus isolation and virus neutralization been run in parallel in this study, herd 4 (table 3.7) would have been correctly classified. Retesting the herds in six months also would have likely led to herd 4 being correctly classified. By then the age cohort of PI heifers would no longer have been in the target population for sampling, yet those heifers in the target population would have been exposed to them and likely

seroconverted. Herd 3 (table 3.7) also would likely have been correctly classified on a herd retest. The PI animal would have been within the target population and its pen mates would have had 6 more months in which to become infected and seroconvert. Both of these methods are more costly, but the increased expense is minimal when compared to whole herd testing.

Issues needing further investigation

There are still some questions regarding the use of serological evaluation of five unvaccinated heifers 6-12 months of age as a method for screening herds for BVDV that were not answered in this study. One of those questions is what is the best definition for a positive herd serological test? The data from this study suggests that the best sensitivity and specificity was obtained defining a positive test as two of five heifers with titers $\geq$ 512. In a previous study, on which the reference cutoff for this study was based, the optimum cutoff for a positive serological test was three of five heifers with titers $\geq$ 128 [23]. Further investigation over a larger study population is needed to better define a positive herd serological test.

Another question that needs to be answered is can serological evaluation of five unvaccinated heifers be used to determine if beef herds are infected with BVDV? To date, this method has only been documented in dairy herds. It seems like it should work for beef herds, but there are some management differences between beef and dairy herds that may influence the test. Beef cattle are generally pastured and the cattle density is somewhat less than in dairy herds. Therefore, a PI animal in a beef herd may not be as efficient at infecting its cohorts as a PI animal in confined dairy housing. Also, due to the

seasonality of breeding and condensed calving season in most beef herds, there is a possibility that beef herds would be more likely to have age cohorts of PI animals that might lead to herd misclassification. This could be avoided by doing virus isolation in parallel with serum virus neutralization or retesting the herd as discussed earlier.

A final question remaining to be answered is can serological evaluation of five heifers be used to screen herds that vaccinate their heifers prior to six months of age? Another herd was tested in this study, but it was not used in the data analysis because it was learned that the heifers sampled had been vaccinated with a modified-live vaccine two months prior to testing. No PI animals were identified in this herd, yet four of the five heifers had BVDV type I antibody titers $\geq$ 1024. In the study in which the best definition for a positive serological evaluation was determined to be three of five heifers with titers $\geq$ 128, herds were separated into three categories. Category A consisted of herds without a vaccination program and without PI animals. Category B consisted of two herds that used killed vaccine and did not have PI animals, while category C consisted of three herds using killed vaccine and which had PI animals [23]. It was unclear if the heifers within the respective target populations were vaccinated prior to being tested. Thus, it appears that the use of a modified live vaccine in animals prior to six months of age would preclude the use of the herd serological test to screen for PI animals. It is uncertain whether killed vaccines would have the same effect, but caution would be warranted when interpreting a positive serological test in such herds.

The question then arises as to how best to screen herds for PI animals in which calves are vaccinated before six months of age, short of extensive individual testing. These herds pose a particular challenge given the disadvantages of bulk tank testing as

well as the problems discerning vaccinated from naturally infected animals on serological evaluation. One option would be to have those herds designate BVDV sentinels, animals that would remain unvaccinated within the herd for at least 12 months. These animals could be steers or free-martin heifers being fed out for beef should the herd not want to risk not vaccinating replacement heifers. The only requirements for these animals would be that they remain unvaccinated and be housed with the rest of the herd.

Conclusion

In short, this study provides evidence that serological evaluation of five unvaccinated heifers 6-12 months of age can accurately identify dairy herds with BVDV PI animals and determine what genotype of the virus is infecting the herd. Thus, it can be a valuable tool in the development of BVDV control and vaccination programs. Issues still needing to be resolved regarding this methodology include better defining a positive serological test, determining whether it can be used in beef herds, and how best to apply it to herds which vaccinate calves before six months of age.

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