Investigation of surveillance techniques to detect *Bovine viral diarrhea virus* in cattle

by

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Abstract

Bovine viral diarrhea viruses (BVDV) are important pathogens of cattle globally. Vaccination is often used to control BVDV, but complete control requires detection and elimination of cattle persistently infected (PI) with BVDV. These cattle are infected with BVDV during gestation, become immunotolerant to their strain of BVDV, and shed large amounts of BVDV throughout their lives. Infections of immunocompetent cattle are transient and produce a variety of clinical signs ranging from unapparent to fatal disease. Cattle PI with BVDV are the reservoir of most transient BVDV infections. Surveillance strategies and diagnostic assays have been developed to identify herds exposed to BVDV and individuals that are PI with BVDV. Many of these strategies and assays are costly and inconvenient for producers and are subsequently underused. New surveillance strategies that fit into common production schemes as well as accurate diagnostic assays based on easily-attainable samples are necessary to improve adoption of BVDV control. The research presented in this paper outlines opportunities to simplify BVDV sampling and detect immune evidence of BVDV infections in the form of nasal antibody specific to BVDV. Specifically, nasal swab samples were found to be as sensitive as the standard ear notch sample used in current antigen capture ELISAs. Alternative skin samples can also be used as accurate diagnostic samples in antigen capture ELISAs provided the kit used for diagnosis has been validated to use those samples. Before any surveillance program based on antibody responses to a pathogen can be implemented, the concentration and duration of passive antibodies to the pathogen must first be established. Research presented in this dissertation demonstrates that antibody of all isotypes can be measured in the secretions associated with the nasal and oral cavities. There is also strong evidence

that passively acquired antibody is transferred transiently into mucosal secretions of the mouth and nose. Analysis of pathogen-specific data reveals that mucosal antibody profiles may prove to be a useful tool for detecting acquired immune responses in calves at a much younger age than is currently permitted by serum antibody analysis. A study designed to investigate the value of BVDV-specific nasal antibodies in calves showed calves with high levels of anti-BVDV nasal antibodies had decreased risk of respiratory disease compared to calves with low levels of anti-BVDV nasal antibodies. These nasal antibodies likely indicate development of a complete, mature immune response to BVDV because of the T-lymphocyte help and time required to produce nasal antibodies specific to BVDV. Overall, the research presented in this dissertation reveals opportunities to improve BVDV detection in the U.S., but more research is needed to validate these techniques and samples.

Chapter 1. Introduction and literature review

Introduction

Bovine viral diarrhea viruses (BVDV) are economically important viruses of cattle worldwide [1]. Cattle can be affected by transient infections with BVDV that result in reproductive loss, immunosuppression, and co-infection with other pathogens [2]. Cattle congenitally infected with BVDV during the first trimester of pregnancy become persistently infected (PI) and serve as the reservoir for new infections [3]. Control of BVDV is based on removal of PI animals from herds to eliminate challenge and vaccination of susceptible animals to prevent transient and congenital infections [4]. Surveys of cattle producers in the United States have shown that most producers recognize BVDV as a significant problem for cattle production, but few are taking action to eliminate BVDV PI cattle [5]. In the same survey, producers reported a lack of return on investment as the primary disincentive for BVDV control. In its current form, detection of BVDV PI cattle requires individual sampling followed by assays that detect the virus. Implementation of individual testing programs requires substantial investment of money and labor.

The overall purpose of the research presented in this dissertation is to mitigate the impact of BVDV infections in cattle. Four specific research objectives are addressed in this dissertation. First, investigation of alternative sample types offers the potential to provide alternatives to those currently in use. Since proficiency testing and validation of diagnostic assays requires large amounts of positive and negative sample types, the

second objective was to evaluate a common assumption that all haired bovine skin can be used as a diagnostic specimen for antigen-based ELISAs. These two objectives were designed and implemented to provide a better understanding of how current BVDV antigen-based diagnostics can be applied.

The third and fourth objectives were designed to utilize a novel approach to measuring immune responses to BVDV. This approach was centered around the concept of quantifying antibodies specific to BVDV on easily accessible mucosal surfaces, particularly of the nose and mouth. The third objective was to quantify the transfer and duration of maternal antibodies to saliva and nasal secretions of neonatal calves. The fourth objective was to determine the impact of BVDV-specific nasal antibodies in calves exposed to BVDV in the field.

These four objectives outline the work necessary to expand the body of knowledge surrounding BVDV management. Improved detection strategies combined with greater knowledge of disease impact will provide tools and incentives for BVDV control.

Dissertation Organization

This dissertation is organized into 6 chapters, including the introductory chapter and the conclusions. Chapters 2-5 and the literature review contained in chapter 1 are manuscripts that are either published in peer-reviewed journals or will be submitted for publication.

Literature Review: Using secretory IgA in nasal secretions to diagnose herds with persistently infected cattle

The purpose of this review is to explore the use of *Bovine viral diarrhea virus* (BVDV)-specific nasal secretory immunoglobulin A (sIgA) as a method immune of surveillance that complements production practices in order to improve producer compliance with BVDV control. *Bovine viral diarrhea viruses* are a group of enveloped, single-strand RNA viruses. BVDVs are classified as members of the genus *Pestivirus* within the family *Flaviviridae* [2]. Two species of BVDV, BVDV1 and BVDV2, have been described [6] and can be divided further in to many subspecies [2]. This marked diversity can be attributed to high mutation rates found in single-strand RNA viruses [7].

BVDV exists as one of two biotypes depending on effect in cultured cells: cytopathic (CP) or non-cytopathic (NCP) [8]. NCP is the predominant BVDV biotype isolated from clinical cases and is the biotype associated with persistent infections (PI). Persistent infections are unique to members of the *Pestivirus* family and occur as a result of congenital infection during development of fetal self-tolerance [3]. Cattle born PI are infected for life, shed large amounts of virus, and efficiently infect non-PI contact cattle [9]. CP viruses arise from mutations of NCP BVDV in PI cattle [10]. Super-infection of a NCP PI animal with CP BVDV results in fatal mucosal disease [11]. Low virulence CP BVDV isolates are commonly included in vaccines because of antigenic similarities with NCP isolates and because they are incapable of producing PI offspring in cattle vaccinated during pregnancy.

BVDV infection results in a diverse array of clinical presentations. BVDV infections in immunocompetent cattle result in immune response and pathogen clearance. These infections are known as transient infections (TI). Transient infections usually result

in subclinical infections causing mild fever, transient leukopenia, and seroconversion [12]. More severe TIs with BVDV cause fever, anorexia, lethargy, alimentary tract ulceration, diarrhea, leukopenia, and lymphoid depletion [13, 14]. Infections with BVDV2 cause particularly severe disease, including pronounced leukopenia, lymphoid depletion, and thrombocytopenia [15]. Transient congenital BVDV infection causes early embryonic death, abortion, and malformations of infected fetuses [16]. BVDV infection has been associated with respiratory disease, both as a primary etiologic agent [17] and in synergistic combination with *Bovine Respiratory Syncytial Virus* [18], *Bovine Herpesvirus-1* [19], *Mannheimia haemolytica* [20], and *Mycoplasma bovis* [21]. This enhanced disease is likely attributable to BVDV-induced immunosuppression [22-24].

BVDV related disease conditions cause significant economic damage. Reproductive losses and clinical illness result in losses ranging from $20-57 per dairy cow year^{-1} [1, 25]. Observational studies coupled with mathematical models designed to predict losses in beef cow-calf production reveal $15-72 loss per cow year^{-1} [26, 27]. Estimations of economic loss in feedlot production can reach $88 per animal exposed to a BVDV PI animal [28]. A majority of cow-calf producers in the U.S. recognize BVDV as a significant problem in the beef industry [5]. Despite the estimated economic losses and awareness associated with BVDV, only 4.2% of surveyed producers tested any calves for PI with BVDV in the three years preceding the survey [5]. Many factors influence producer decisions to diagnose and removed BVDV PI calves from their herds including perceived return on investment, negative results of previous tests, and labor costs.

Because producers view BVDV as a significant issue, vaccination is a commonly implemented control measure. While vaccination offers considerable protection from the

harmful effects of BVDV infection [29, 30], evidence of vaccine failure exists [31]. Removal of PI animals to control challenge is needed to adequately control BVDV infections. Programs aimed at controlling or eradicating BVDV use a variety of diagnostic techniques with or without vaccination [32]. In the U.S., where vaccination is common, BVDV diagnosis centers on agent detection. Specifically, immunohistochemistry and enzyme-linked immunosorbent assays target protein antigens of BVDV [33, 34] and reverse-transcriptase polymerase chain reaction targets highly conserved regions of the BVDV genome [35]. These assays are accurate [36, 37], but require individual testing of all at-risk animals as well as follow-up testing to rule out accidental diagnosis of TI. Individual and follow-up testing are expensive and negative results are common given the low prevalence of PI cattle [38]. This scenario, coupled with insidious damage associated with BVDV infections, often leads producers to perceive a poor return on investment in PI surveillance.

In areas where BVDV control has become organized, detection of immune responses to BVDV infections is used to limit whole-herd agent testing to herds with evidence of infection [39]. Detection of immune responses, usually BVDV-specific antibody, is advantageous in control programs for several reasons. First, antibodies against BVDV have been shown to cross-react with distantly related BVDV isolates as well as other *Pestiviral* species [40]. Antibody cross-reactivity allows detection of BVDVs that are not directly targeted by the diagnostic assay. Reports of false negative antigen assay results highlight the value of assays that detect antibody cross-reactivity [41]. Second, sentinel populations can be monitored for serum antibody responses to BVDV rather than all individuals in a herd [42]. These sentinel populations are usually

groups of unvaccinated, young animals that no longer have BVDV-specific maternal antibody and have had adequate opportunity to become exposed and seroconvert to BVDV. Sentinel surveillance allows diagnosis of circulating BVDV infections using a small percentage of the herd to provide evidence justifying whole-herd agent testing to detect PIs. The limitations of sentinel-based immune surveillance using serum antibodies are revealed by the selection criteria. The presence of maternal antibody prevents accurate designation of status, as does the development of serum antibody following vaccination [39]. One study demonstrated successful herd-level diagnosis by measuring pre-colostral, BVDV-specific, serum antibodies in dairy calves [43]. This study effectively negated the confounding influence of maternal antibody and vaccination by choosing gestating calves as the sentinel population. While this approach was successful, adoption is limited by the requirement of precolostral serum samples, a difficult task in extensively managed beef cattle. The fourth advantage of using immune surveillance to detect BVDV infections is the ability to use samples other than serum. Bulk tank milk samples can easily be screened for BVDV-specific antibody [44]. This strategy is particularly useful to monitor status in herds that are naïve and do not vaccinate [39]. However, naïve, unvaccinated herds are very uncommon in the U.S. Herds with PI exposure will maintain high levels of bulk milk antibodies as long as exposed animals remain in the herd, thereby limiting the utility of this strategy early in BVDV control efforts.

Currently, BVDV control in the U.S. is limited by producer compliance, economic factors, and limited availability of useful surveillance strategies that fit production practices. A method of BVDV surveillance is needed that removes these

limitations by providing a simple, cost-effective, production compatible method. The goal of BVDV control is to mitigate new infections through removal of the source, the PI bovine. Conclusive diagnosis of PI status will continue to rely on BVDV antigen or genome detection; however, use of immune surveillance can be used to limit individual animal testing to groups of animals with evidence of exposure.

Secretory IgA holds promise as a means of diagnosing infections of the mucosal surface, as is the case with BVDV, because of the targeted, active process of sIgA production and action. Currently, specific information related to induction, production, and function of sIgA in the bovine nasal passages is scarce. In contrast, significant research has been published on this topic in regard to humans and laboratory animals and in particular, the gut associated lymphoid tissue of these species. While the current body of knowledge must be confirmed by experiments examining the lymphoid tissue associated with the upper respiratory tract of cattle, current understanding of mucosa-associated immunity will be used to postulate the value of BVDV-specific sIgA surveillance.

Mucosa-associated lymphoid tissue (MALT) can be observed on every major bovine mucosal surface including the respiratory, alimentary, and genitourinary tracts [45]. These aggregations of lymphoid tissue specialize in detection and response to antigens encountered on each respective mucosal surface. Although MALT tissues may be physically distant from one another, they are linked together as part of the common mucosal immune system (CMIS) [46]. The CMIS has been practically demonstrated in mice by measurement of sIgA responses in intestinal and genital tracts following intranasal immunization with *Respiratory Synsytial Virus*-containing immune stimulating

complexes [47]. Similarly, calves challenged with *Bovine Respiratory Synsytial Virus* by a combined intranasal and intratracheal route showed IgM and IgA responses in feces and saliva in addition to IgM and IgA in nasal secretions and lung lavage fluid [48].

Secretory IgA-producing plasma cells are activated in MALT tissues as a result of immune surveillance of mucosal surfaces. The process begins as specialized follicle-associated epithelial cells, known as microfold cells (M-cells) transcytose luminal contents by clathrin-mediated endocytosis, fluid-phase pinocytosis, actin-dependent phagocytosis, and macropinocytotic engulfment [49]. Immediately deep to the M-cell, clonally expanded B-lymphocytes are thought to survey transcytosed antigens and present them on MHCII to adjacent T-lymphocytes [50]. In mice, B-lymphocytes that bind their cognate antigen have been shown to receive co-stimulatory and cytokine, namely TGF-β, IL-5, IL-6, and IL-10 help to induce proliferation and class switching to predominantly IgA-producing plasma cells [51]. These plasma cells leave the MALT in which they were activated and respond to homing signals that result in migration to mucosal lamina propria or to sites of mucosal inflammation [51].

Mature, IgA-producing plasma cells in murine lamina propria translate monomeric IgA in the Golgi apparatus and then assemble polymeric, J-chain-linked IgA during secretion into the interstitial space [52]. Polymeric IgA diffuses through interstitial fluid to arrive at the basolateral surface of mucosal epithelial cells. Bovine mucosal epithelial cells have been shown to express polymeric immunoglobulin receptors (pIgR) that specifically bind to the J-chain associated with polymeric IgA [53]. Polymeric immunoglobulin receptors facilitate endocytosis of polymeric Ig including IgA, translocation to the apical cell surface, and exocytosis onto the mucosal surface. On the

mucosal surface pIgR is cleaved to release secretory IgA, a complex protein composed of J-chain linked polymeric IgA bound to a remnant of pIgR known as secretory component (SC) [54]. In addition to its critical role in polymeric IgA transport to mucosal surfaces, SC minimizes proteolytic cleavage of sIgA on the mucosal surface [55] and binds to mucin in mucosal secretions allowing longer activity at the mucosal surface and promoting pathogen clearance via the mucociliary apparatus [56].

Immune surveillance using mucosal antibodies is already commonly practiced in other areas of human and veterinary diagnostics. Human oral fluids are routinely collected and assayed for the presence of anti-HIV antibodies as a screening technique to identify new HIV infections [57]. Similarly, oral fluids are collected from groups of pigs to track viral infections such as *Porcine respiratory and reproductive syndrome virus* and *Porcine circovirus 2* [58, 59]. In oral fluid samples, sIgA is present in combination with other isotypes of antibodies including IgG and IgM. Each of these antibodies has been shown to arrive in oral fluid following intravenous administration [60]; however, in healthy tissue the majority of oral antibody is produced locally with the dominant isotype being IgA [61]. Antibodies of the IgG isotype are thought to arrive in the healthy oral cavity as part of a crevicular serum transudate from gingival pockets [62]. During episodes of mucosal inflammation, increased vascular permeability is likely to allow significant amounts of serum Ig to bathe mucosal surfaces. This concept is supported by evidence in rats that shows increased movement of IgG into exudates in inflamed air pouches [63]. While the presence of any pathogen-specific antibody may suffice as evidence of natural infection for many diseases, the complexity of BVDV control in the U.S. requires measurement of antibody that can only be attributable to natural infection.

Because of this constraint, any measurement of IgG in mucosal fluid may result in false positive surveillance results due to the presence of serum IgG attributable to maternal antibody transfer or parenteral vaccination.

Secretory IgA offers unique opportunities for disease surveillance because of its biology in mammals. First, sIgA is likely to be produced on mucosal surfaces distant to the one exposed to BVDV because of the circulation of plasma cells through the CMIS to mucosal effector sites. While BVDV is most often acquired via the oronasal route, cattle can also become infected by exposure of genital mucosal surfaces [64]. Mucosal samples can be easily collected from mucosal surfaces of the head and nasal secretions contain high concentrations of sIgA compared to oral secretions [65]. Since BVDV immune surveillance would likely employ nasal secretion samples, plasma cell migration following genital exposure to BVDV may be detectable. Second, mucosal sIgA specific to BVDV may allow differentiation of vaccinated from naturally infected cattle. Previous research demonstrated that parenteral priming of cattle with live *Bovine respiratory syncytial virus* (BRSV) failed to induce mucosal antibody responses [48]. Currently, most BVDV vaccines are administered via the parenteral route. Little is known about the impact of route of exposure on mucosal sIgA production in cattle, but given similar behavior to BRSV, vaccinates would only have nasal sIgA if they had previously been mucosally infected with live BVDV. Third, animals PI with BVDV tend to cause infections and antibody production in large percentages of the population in which they are housed [9]. Because of the high rate of infections seen as a result of PI exposure, relatively few nasal samples would be required to diagnose herd exposure status with high confidence [66]. A fourth benefit of nasal sIgA testing is the ability to predict risk of

BVDV-related disease conditions based on demonstrated immunity. Significant protection from bovine respiratory disease has been shown to be significantly related to high levels of nasal antibody specific to BVDV [67].

While BVDV-specific sIgA may serve as a plausible herd screening method, several knowledge gaps must first be filled to validate the technique. First, mucosal immune responses following both parenteral and mucosal exposure to BVDV vaccines, either modified-live or inactivated, have not been described in the literature. This information is essential to the accuracy of any diagnostic assay developed to measure sIgA in nasal secretions of cattle as well as to the ability to differentiate between parenteral vaccinates and cattle naturally exposed to BVDV. Second, mucosal antibody responses are thought to be short-lived and research investigating the duration of nasal sIgA response to BVDV is necessary for effective implementation of nasal sIgA-based herd screening. [68]. Some long-lasting nasal sIgA responses have been documented in cattle [69] and studies in mice and rats have shown that long-lasting mucosal sIgA responses can be developed, especially with repeated delivery of antigen [70, 71]. While yet unproven, BVDV infection is likely to produce long-lasting nasal sIgA responses due to the chronic nature of exposure from PI animals. Third, epidemiologic parameters related to the prevalence of detectable sIgA responses in groups of cattle with PI animals, TI animals, and vaccinates are necessary to establish appropriate sample sizes for herd level screening. Based on previously cited serologic evidence, sIgA prevalence associated with PI animals is also likely to be high. Future studies will need to determine the prevalence of BVDV-specific sIgA as a result of PI exposure.

BVDV continues to infect cattle and inflict financial damage on producers. Development of new surveillance tools that fit current production schemes is an important step toward better control. Future research examining the impact of BVDV infection and vaccination on mucosal antibody production may provide the foundation necessary to develop these diagnostics.

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Chapter 2. Comparison of detection of *Bovine virus diarrhea virus* antigen in various types of tissue and fluid samples collected from persistently infected cattle

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Brian VanderLey, Julia Ridpath, Shaun Sweiger

Abstract

Bovine viral diarrhea viruses are economically important pathogens of cattle. Most new infections are acquired from animals persistently infected with the virus. Surveillance programs rely on skin biopsies for detection of persistently infected (PI) cattle. The purpose of this study was to compare antigen capture enzyme-linked immunosorbent assay (ACE) testing results using different types of samples from PI animals. The intent was to determine comparative detection rates in types of samples that are frequently submitted to diagnostic laboratories for evaluation of cases of unknown etiology or samples that could be easily collected for *Bovine viral diarrhea virus* (BVDV) screening. Eight types of samples were collected from 40 PI animals. The sample types were ear notches, serum, nasal swabs, conjunctival swabs, oral swabs, rectal swabs, vaginal/preputial swabs, and a tail-skin fold biopsy. Each type of sample (n = 8) for each animal (n = 40) was evaluated with a commercial ACE kit. When using ACE, tail-skin fold and nasal swab samples were 100% sensitive compared to results using ear notches. Sensitivity using other samples was as follows: serum and vaginal/preputial swabs, 92%; conjunctival swabs, 64%; rectal swabs, 10%; and oral swabs, 8%. Testing of tail-skin fold biopsies, nasal swabs, and ear notch samples resulted in reliable results. In

contrast, other sample types were unreliable for diagnosis of persistent infection in calves.

Introduction

Exposure to *Bovine viral diarrhea virus* (BVDV; family *Flaviviridae*, genus *Pestivirus*) results in economically important diseases in cattle.[5,6] Infection with BVDV results in both persistent (a result of in utero exposure) and acute (a result of postnatal exposure) infections.[4,7] Although viral shedding from acutely infected animals has the potential to infect other animals, persistently infected (PI) animals can shed high levels of BVDV during their lifetimes and are thought to be the principal source of new infections. An important strategy for controlling the economic impact of BVDV is to test for and remove PI animals. Several testing strategies have been developed to identify BVDV-infected animals, and more importantly, differentiate acutely infected animals from those that are persistently infected. Examples of these tests include virus isolation, polymerase chain reaction (PCR), and immunohistochemistry (IHC). Antigen capture enzyme-linked immunosorbent assay (ACE) has also been developed to detect viral antigen that is present in either tissues or serum. The ACE-based tests are robust, economical methods of identifying PI cattle and are the most rapid tests available to date.[2] The purpose of the current study was to investigate the potential of other types of samples for identifying cattle persistently infected with BVDV. The intent was not to validate alternative samples for BVDV detection, but rather to determine diagnostic sensitivity of samples that are frequently submitted to diagnostic laboratories for evaluation of cases of unknown etiology (e.g., cases of respiratory disease outbreaks in cattle) or types of samples that could be easily collected for BVDV screening.

Materials and methods

Eight types of samples were collected from 40 animals previously identified as persistently infected based on 2 positive ACE tests of ear notch samples collected at least 10 days apart (OIE Manual of Diagnostic Tests and Vaccines for Animals Chapter 2.10.6; www.oie.int/eng/normes/mmanual/A_00132.htm). Three animals were from a stocker operation in northeastern Oklahoma (Washington County) and 37 were from a stocker operation in south central Oklahoma (Pottawatomie County). All of the animals were segregated from other animals on each premise because of their BVDV infection status. The 8 types of samples collected were as follows: ear notch and caudal tail fold biopsies, serum, and swabs from the prepuce or vagina, nasal cavity, ocular conjunctiva, oral cavity, and rectum. Both the ear notch and caudal tail fold samples were obtained with a small ear notcher that was cleaned with dilute chlorhexidine solution and rinsed in clean water between each calf. Serum samples were obtained by collecting whole blood via jugular venipuncture. Vaginal and/or preputial, nasal, conjunctival, oral, and rectal swabs were obtained by placing a polyester-tipped plastic applicator[a] in contact with the sampled mucosal surface and rotating the swab rapidly several times. In situations where bilateral sampling sites were available (conjunctival swab, nasal swab), only one side was sampled.

After collection, swabs were immediately submerged in 1 ml of sterile phosphate buffered saline (PBS) and placed on ice. Ear notch and caudal tail fold samples were placed in dry tubes and held on ice for approximately 2 hr for transport to the laboratory. Both skin and swab samples were frozen at –20°C immediately upon arrival at the lab.

The blood collected from each animal was centrifuged at 750 × *g* for 20 min, and the serum was placed in sterile 15-ml tubes and frozen at –20°C. Twenty-four hours before testing, swab samples were thawed at 4°C. Twenty-four hours prior to testing, 2 ml of PBS was added to each ear notch and caudal tail fold samples. After addition of PBS, samples were held at 4°C until testing. Serum samples were thawed immediately prior to testing.

A commercially available ACE test[b] was used to evaluate the samples for the presence of BVDV E^{rns} (gp48) antigen.[9] ACE testing was performed per manufacturer's directions. Optical density (OD) readings were determined, and the presence or absence of BVDV antigen in each sample was determined by calculating the sample to positive (S/P) ratio for each sample per manufacturer's directions. The calculation of the S/P ratio was determined using the following formula:

$$S/P = (Sa – N)/(Po – N),$$

where Sa is the sample OD, N is the mean OD of the negative control, and Po is the mean OD of the positive control. Guidelines provided with the kit state that an S/P ratio of less than 0.20 indicates a negative BVDV antigen status, while an S/P ratio greater than 0.39 indicates a positive BVDV antigen status. Samples with S/P ratios between 0.20 and 0.39 are graded as "suspect" for BVDV antigen.

In addition to testing each type of sample by ACE, a second ear notch from each calf was submitted to the Iowa State University Veterinary Diagnostic Lab (Ames, Iowa) for BVDV IHC.[8] Serum virus neutralization assays were completed using BVDV virus from the subgenotypes 1a, 1b, and 2a. The BVDV titers regressed against S/P ratios for each sample type were evaluated using a statistical analysis software package.[c]

Results

Results from testing each sample type were compared to the ACE and IHC testing results for each of the ear notch samples. Of the 40 calves tested, 39 were positive by the ACE and 40 were positive by the IHC. Because this investigation is principally concerned with ACE, results will be expressed as sensitivity based on the assumption that calves detected as persistently infected by ACE are true positives (see Fig. 1). The ACE test results were 100% sensitive using tail biopsies and nasal swabs. The sensitivity of ACE was reduced for the other sample types. The ACE results were 92% sensitive for detecting BVDV using vaginal and/or preputial swabs and serum; 64% sensitive using conjunctival swabs; 10% sensitive using rectal swabs; and 8% sensitive using oral swabs. Average BVDV titers were linearly related to S/P ratio for rectal and nasal swabs. No significant relationship was shown between BVDV titer or any other sample type (see Table 1).

Discussion

Although the prevalence of BVDV PI animals in the general population in North America is less than 1%, studies have shown that 7% of animals dying from infectious diseases in feedlots are persistently infected.[3] Further, mortality rates among PI animals are 10-fold higher than those observed in non-PI animals.[5] Skin biopsies, serum, and buffy coat samples are the sample types most frequently collected for surveillance efforts, and commercial test kits have been developed based on testing of these sample types. However, collection of skin biopsies is invasive and can be potentially disfiguring. Further, skin biopsies, serum, and buffy coat samples these sample types are not always submitted in cases of disease outbreaks of unknown etiology. The purpose of the current

study was to investigate the potential benefit of other types of samples for identifying cattle persistently infected with BVDV. The sample types used in the present study were selected based on similarity to standard sample types (tail fold biopsy), accessibility (nasal, conjunctival, oral, rectal, and vaginal swabs), comparability (preputial swabs on males for comparison to vaginal swabs on females), and because samples could otherwise be submitted in respiratory outbreaks of unknown etiology (nasal swabs).

The current study revealed that the test results of skin biopsies from the caudal tail fold and nasal swabs had a high sensitivity compared to the standard ear notch sample. In contrast, testing of vaginal, preputial, conjunctival, oral, and rectal swabs performed at unsatisfactory levels. While the exact reason for the diminished performance of the vaginal, preputial, conjunctival, oral, and rectal swabs is undetermined, it is suspected that magnitude of viral shedding and bacterial contamination can potentially play a major role in each of the cases. Oral and rectal swabs, both from sites with very high bacterial loads, performed very poorly compared with nasal swabs or ear notches. Vaginal, preputial, and conjunctival swabs were taken from sites that may have had lower levels of viral shedding than in other tissues. Simple linear regression of serum antibody titer versus S/P ratio for each sample type was done to determine if the presence of antibodies affected detection by ACE (see Table 1). Serum antibody titers against BVDV demonstrated a significant linear relationship with S/P ratios from nasal and rectal swab samples. While nasal samples remained sensitive despite decreasing S/P ratios with increasing titers, sensitivity associated with rectal swabs may be more affected by increased antibody titer. The presence of serum antibody titers did not significantly affect the S/P ratios of other tissues tested. Such observations require further investigation. It

should be noted, however, that inclusion of suspect animals substantially increased the sensitivity of several sample types. The result indicates that virus may be successfully detectable at a low level in sample types other than skin and nasal swabs. The use of nasal swab samples has several potential benefits. Nasal swabs are a commonly submitted sample type for screening for a number of respiratory pathogens. Because BVDV often plays a role in the development of respiratory disease, the ability to use one sample type for several different tests would be beneficial in terms of both time and economics.

One animal in the current study tested positive by IHC but was negative by all forms of ACE. A previously published report describes a scenario in which 3 acutely infected calves tested positive at initial screening by ACE and by IHC.[1] The calves remained IHC positive 90 days after initial test, but were negative by all other tests including ACE, PCR, and virus isolation. The results from the study indicate that some BVDV antigens may persist in skin tissues after ACE detection is no longer possible.[1] Such a scenario could explain the apparent discrepancy between ACE and IHC in the current study.

As stated above, another advantage to different sample types is the ability to choose a non-disfiguring sample for testing show animals. A significant potential source of BVDV spread is possible if a PI animal is brought to a livestock show and commingled with many other animals. The risk is especially high if pregnant animals are present. Required testing prior to livestock showing events is becoming more common, but owners are frequently unwilling to have an ear notch collected because of the disfigurement of the ear. While the collection of a serum sample is not disfiguring, the

accuracy of testing serum samples, as determined in the present study, was lower than nasal swabs. A non-disfiguring, noninvasive, more accurate method of sampling, such as nasal swab sampling, would likely increase compliance with testing and reduce the transfer of BVDV at show events.

Sources and manufacturers

a. Puritan Medical Products Co. LLC, Guilford, ME.

b. IDEXX Laboratories Inc., Westbrook, ME.

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Tables and figures

Table 1. Comparison of affect of virus neutralizing titer on the sample to positive ratio of each sample type.

Sample	Slope	P-value
Conjunctival swab	–0.0089	0.6553
Ear notch	–0.0041	0.8895
Nasal swab	–0.0833	0.0115*
Oral swab	–0.0058	0.4592
Rectal swab	–0.0378	<0.0001*
Serum	–0.0192	0.2217
Tail biopsy	–0.0173	0.4529
Vaginal and/or preputial swab	–0.0326	0.2895

* Statistically significant at the $p < 0.05$ level.

Figure 1

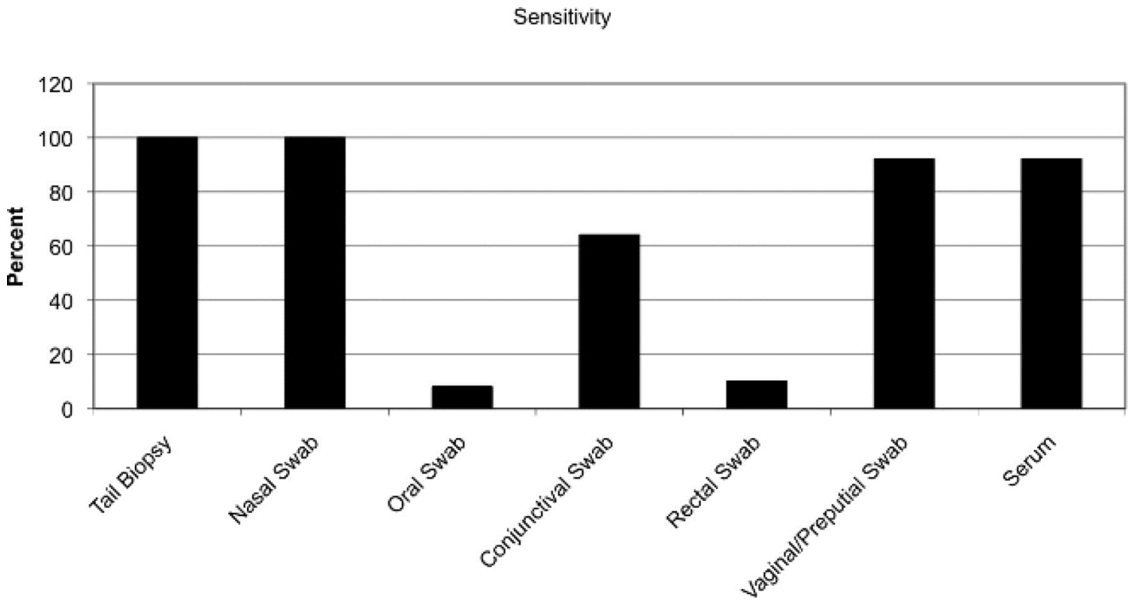

Figure 1. Relative sensitivity of each sample type compared with antigen capture enzyme-linked immunosorbent assay on ear notch samples.

Chapter 3. *Bovine viral diarrhea virus* antigen detection across whole cattle hides using two antigen-capture enzyme-linked immunosorbent assays

A paper published in *The Journal of Veterinary Diagnostic Investigation*

Brian L. Vander Ley, Julia F. Ridpath, Shaun H. Sweiger

Abstract

Bovine viral diarrhea virus is a costly disease of cattle that can be controlled by vaccination, biosecurity, and removal of persistently infected cattle. Development and proficiency testing of assays to identify persistently infected cattle requires substantial quantities of known positive- and negative-sample material. The objective of this study was to determine what sections of bovine skin contained Bovine viral diarrhea virus antigen. Two commercially available antigen-capture enzyme-linked immunoassays were used to test subsamples representing the entire skin of 3 persistently infected calves. Both assays detected *Bovine viral diarrhea virus* antigen in the samples indicated for use by assay protocol. However, one assay identified all subsamples as positive, while the second assay identified 64.4% of subsamples as positive. These results show that use of samples other than those specified by the assay protocol must be validated for each individual assay. In this study, alternative sample sites and use of the entire hide for proficiency testing would be acceptable for only one of the assays tested.

Introduction

Bovine viral diarrhea virus (BVDV; family *Flaviviridae*, genus *Pestivirus*) is a costly disease of cattle in the United States and around the world.[2,3] The main reservoirs of BVDV in cattle populations are cattle persistently infected (PI) with BVDV due to

early gestational exposure.[5] Persistently infected calves continuously shed large amounts of BVDV. Testing and removing PI calves from herds is useful for controlling spread of BVDV and is economically beneficial. Currently, several diagnostic tests are available for detecting BVDV PI calves including antigen-capture enzyme-linked immunosorbent assays (ACEs), which detect antigen in skin and serum samples. Ear notch samples are frequently used for BVDV detection and have been used in the licensing of several tests. However, development and validation of ACEs requires substantial quantities of known positive and negative sample material and the amount of ear skin samples that can be harvested from 1 animal is limited. Further, it is desirable to develop panels of known samples to be used in laboratory proficiency testing. While it is commonly held that all haired skin from PI cattle contains BVDV antigen, very little work has been done on skin samples from locations other than ears. The purpose of the current study was to use 2 different ACEs to characterize the availability of BVDV antigen present in the total hide of cattle and to determine if skin from various regions of the body is suitable for validation and proficiency testing.

Materials and Methods

Three 200–300 kg cross breed beef calves, determined to be persistently infected by ACE,[a,8] were used in the present study. Each calf was humanely euthanized by ranch personnel according to ranch protocols. The entire hide was removed from each calf, including the hide on the head and legs down to the coronary bands. At the time of harvest, the hides were divided into nearly equal quarters by folding them in half front to back and cutting on the crease and then folding them in half side to side and cutting on the crease again. Each quarter was labeled with the calf's ID and a description of the

quarter (FL, FR, RL, and RR indicating front left, front right, rear left, and rear right, respectively) by placing a labeled ear tag in the quarter. Each quarter was then placed in an individual bag and placed on ice for transport to the lab. Quarters were stored at –20°C.

Each quarter was sectioned into 10 cm × 10 cm squares starting at the corner of the quarter nearest the middle of the hide. Sectioning was done using a precut 10 cm × 10 cm template and common utility knives. Each section was given a unique identifying code to facilitate reconstruction of data maps based on location for analysis. The code consisted of a 2 letter code to identify the quarter (previously mentioned), a number to identify the medial to lateral position, and a letter to identify the cranial/caudal location. Each section was subsampled using a 4-mm leather punch that removed a full thickness circular sample of skin. The punch was cleaned with alcohol and then rinsed with water between each sample. Each section of hide was placed in a resealable bag and stored at –20°C. Two samples were collected from each section and placed in 1.5-ml microcentrifuge tubes. The tubes were also stored at –20°C prior to ELISA analysis.

Samples from each skin section were analyzed for BVDV antigen using 2 different commercial ACE kits (A^a and B^b). One subsample from each section was used for each kit. Each kit was used according to the manufacturer's protocol. Samples for assay A were soaked in phosphate buffered saline (PBS), and an aliquot of the PBS was assayed for the presence of E^{rns}, a structural protein of BVDV.[8] Samples for assay B were soaked in the extraction buffer provided by the manufacturer, and an aliquot of this buffer was assayed for the presence of NS2/3, a nonstructural protein of BVDV. Since multiple plates from each kit were used to analyze all samples, great care was taken to make sure

that each plate was treated exactly the same way. Incubation times, wash volumes, and procedure order were identical for each plate. Sample optical density (OD) was measured using a plate spectrophotometer. The sample-to-positive (S/P) ratio was calculated using the following equation.

$$S/P = (\text{sample OD} - \text{negative control OD})/(\text{positive control OD} - \text{negative control OD})$$

The OD values of assay B were standardized using percent positivity (PP) according to protocol instructions, and PP was calculated using the following equation.

$$PP = (\text{sample OD}/\text{positive control OD}) \times 100$$

The S/P ratio and PP data were used to construct sample signal intensity maps based on hide location.

Data from each kit type was analyzed with a statistical analysis software package.[c] Frequency tables were constructed to show test results by quarter. Prior to analysis, S/P ratio and PP data were examined for normal distribution. If data were not distributed normally, log transformation was employed to achieve normal distribution. Because of variability seen in assay B results, an examination of the relationship between skin thickness and logPP was conducted.

Results

Assay A returned positive S/P ratios for samples from all sections from each calf. Assay B identified 360 (64.4%) of 618 sections as positive and 220 (35.6%) as negative. Table 1 shows positive and negative results by quarter for assay B.

The PP data from assay B were right skewed and were log transformed. The results of a linear regression of skin thickness with logPP demonstrated that no significant linear relationship was present.

Discussion

The data in the current study show that BVDV antigens are present throughout the skin of animals persistently infected with BVDV. In the case of assay A, all skin sections returned a positive result, and overall variation in S/P ratio did not result in erroneous test conclusions for any particular section. Results from assay B demonstrated substantial variability to the point of misclassifying 35.6% of the skin sections. It is important to note that all skin sections that included the ear were positive using both assays. Visual examination of maps of assay B results did not demonstrate a definitive pattern of positive and negative results.

Reasons for the discrepancies between assays remain unknown. One major difference between the 2 assays is their detection target. Assay A targets the E^{rns} protein of BVDV. This protein is associated with the viral envelope and host cell membranes and appears to be readily released into extracellular fluid.[6,7] Assay B targets an epitope of the NS2/3 protein, which has been reported as a successful ELISA target for use in identifying animals persistently infected with BVDV.[1] The antibodies found in assay B have also been incorporated into a blocking ELISA as a means of capturing p80 to serve as a target antigen to measure antibodies resulting from BVDV infection.[4] The NS2/3 protein is localized within host cells and is not a structural component of the BVDV virion.[6] Because of its location, NS2/3 requires more intensive extraction methods to

make it available for detection. This difference in antigen location and availability may explain some of the differences in response seen between the 2 assays.

The differences in results between the 2 commercial ACE kits examined in the current study reveal the need to validate sample site for any particular BVDV antigen test. In the case of assay A, skin samples from any piece of haired skin were found to be adequate samples for PI status determination as well as proficiency testing. However, skin samples other than the ears returned highly variable results when using the assay B kit. This variability would make using any alternative samples for PI determination or proficiency testing unreliable.

Overall, both tests identified the calves as BVDV positive when using the sample (ear notch) indicated for use by kit instructions. In the present study, assay A was a more robust test for identifying BVDV antigen in skin samples other than ear notches. The use of parts of hides or entire hides for proficiency sampling will still require validation of desired sample sources on an individual assay basis.

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Sources and manufacturers

a. Herdchek Bovine Viral Diarrhea Virus Antigen Detection Kit. IDEXX Laboratories Inc., Westbrook, ME.

b. Priocheck BVD Ag PI. Prionics AG, Zurich, Switzerland.

c. SAS Institute Inc., Cary, NC.

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Tables

Table 1. Number of *Bovine viral diarrhea virus*–positive and –negative sections for each cattle hide quarter along with the corresponding percentage for that quarter using the assay B kit.

	Total		Calf 1		Calf 2		Calf 3	
Quarter	**Positive**	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**
Front left	77 (55)	62 (45)	28 (53)	25 (47)	30 (61)	19 (39)	19 (51)	18 (49)
Front right	70 (50)	69 (50)	28 (51)	27 (49)	21 (53)	19 (48)	21 (48)	23 (52)
Rear left	126 (75)	42 (25)	51 (84)	10 (16)	43 (78)	12 (22)	31 (55)	25 (45)
Rear right	125 (73)	47 (27)	61 (94)	4 (6)	36 (71)	15 (29)	29 (56)	23 (44)

* Numbers in parentheses are percentages. Assay B was manufactured by Prionics AG, Zurich, Switzerland.

Chapter 4. Quantification of maternal antibodies transferred to the mucosal surface of neonatal bovine calves

Brian L. Vander Ley, Shaun H. Sweiger, James A. Roth, and Julia F. Ridpath

Abstract

Mucosal antibodies are an important defense mechanism against invading pathogens as well as an increasingly popular target for disease surveillance. Neonatal cattle receive passive antibodies following ingestion of colostrum. While some reports have shown the presence of colostral antibodies in mucosal secretions of young calves, the purpose of this study was to comprehensively document the transfer of colostral antibodies to saliva and nasal secretions of neonatal calves. Eight calves were enrolled in the study by observing parturition and removing the calf prior to colostrum ingestion. Pre and post colostral saliva and nasal secretions were collected and assayed for IgM, IgA, IgG1, and IgG2 as well was for IgA specific to *Bovine viral diarrhea virus*. This study demonstrates significant evidence of maternal antibody transfer to the oral and nasal mucosal surfaces of calves. IgA, the most abundant mucosal antibody isotype, demonstrated a clear pattern of transfer that could not reasonably be attributed to any other mechanism due to the time required by a calf to mount an IgA response. Additionally, pathogen-specific antibody profiles demonstrate that mucosal antibodies may serve as a useful tool for identifying acquired immune responses in cattle at a younger age than is permitted by serum antibody analysis.

Introduction

Mucosal antibodies (MA) are an important component of protective immune responses to pathogens that gain access to hosts via a mucosal surface. MA protect hosts by trapping pathogens in the mucous layer, by binding pathogen attachment sites to prevent invasion and by neutralizing toxins [1]. Cattle are susceptible to a variety of pathogens that gain access via mucosal surfaces. Of these, respiratory pathogens are particularly detrimental to cattle production [2]. Immunoglobulin transfer from dam to offspring has been shown to protect neonatal calves from respiratory disease [3], and other reports have shown that maternal antibody is transferred to oral and nasal mucosal surfaces of neonatal ruminants [4-6]. However, these reports present differing conclusions regarding the major types of immunoglobulins that are transferred to oral and nasal mucosal surfaces of neonatal calves. A comprehensive understanding of transfer of MA in neonates is necessary to understand the nature of MA kinetics from birth and to implement management strategies that exploit protective and diagnostic opportunities afforded by measurement of MA. The research presented in this report elucidates the transfer of maternal antibody to oral and nasal mucosal surfaces of neonatal calves.

Materials and Methods

The animal methods used in this project were approved by the Iowa State University Institutional Animal Care and Use Committee.

Eight Holstein calves (4 male, 4 female) born in a 24 hour period were enrolled in the study. Parturition was observed for each enrolled calf to insure that no colostrum ingestion occurred prior to initial sampling. Immediately following parturition, calves were removed from the dam, dried and identified using plastic ear tags with unique

numbers. A mucosal secretion collection device consisting of a modified 1 ml serologic pipette connected in series to a red top Vacutainer tube and then to a vacuum pump was used to collect mucosal fluid from both the oral and nasal mucosal surface of each calf. Ear notch samples and whole blood were also collected from each animal.

Following initial sample collection, calves were processed according to farm protocol. Each animal received 1 gallon of colostrum from a pool of sufficient size to enable feeding of all 8 calves from the same pool. The colostrum was administered via an esophageal feeder. The umbilical stump of each calf was treated with an iodine solution. Each calf received a bolus containing immunoglobulin against *Escherichia coli* K99 and bovine coronavirus (First Defense) and a subcutaneous injection of anti-colostridial toxin serum of equine origin (Clostridium C and D antitoxin, Colorado Serum Company). All calf processing procedures listed were performed within an hour of birth.

Saliva, nasal secretions, and whole blood samples were collected on day 0 and then again on days 2, 7, and 14. Samples were collected prior to the morning feeding to allow as much time as possible for milk replacer associated immunoglobulins to dissipate. Whole blood was allowed to clot and then was centrifuged for 20 minutes at 1000 x *g*. Separated serum was decanted into storage tubes. All samples stored at -20ºC prior to analysis.

All calves in the study were moved to individual calf housing within 12 hours of birth. Each calf stall consisted of a wire panel enclosure with straw bedding that allowed nose to nose contact with neighboring calves. Calf enclosures were part of a passively ventilated (curtain) barn and all study calves were housed in the same section of the barn. Throughout the trial, calves were fed 2 quarts of reconstituted powdered milk replacer

twice daily. Calves were also offered starter feed and water for the duration of their stay in individual housing.

Nasal and oral samples were diluted with phosphate buffered saline containing 1mM EDTA (PBS-EDTA) at a ratio of 1 part sample to 3 parts PBS-EDTA. An equal volume of dilute dithiothreitol (Sputolysin reagent) was added to each diluted sample. After vortexing, the samples were incubated at room temperature for 15 minutes followed by a 3 minute centrifugation step at 3000 *x g*. Processed samples were either used immediately or stored at -80ºC.

Total isotype specific immunoglobulin assays were used to quantify antibody concentrations in each sample collected from the calves. ELISA kits (Bethyl Laboratories) were used to quantify IgA, IgG1, IgG2, and IgM according to the standard reference sample provided with the kit. Isotype-specific immunoglobulin concentrations were determined from the standard reference sample using a microplate reader and accompanying software (Molecular Devices Softmax Pro). Samples were diluted with recommended buffers so that resulting signals fell within the range of signals developed by the reference sample standard curve.

BVDV-specific IgA was measured in serum, nasal secretions, and saliva using a BVDV type 1 antibody detection ELISA kit (Idexx). The BVDV antibody ELISA kit was modified by the addition of anti-bovine IgA antibody conjugated with HRP at a dilution of 1:40,000 (Bethyl Laboratories). The BVDV antibody ELISA was used according to kit instructions for all samples except that substrate incubation time was increased from 10 minutes to 20 minutes at room temperature. Raw optical density (OD) results were

normalized by use of the following equation. Sample to positive ratio (S/P) = [(Sample OD-Negative Control OD)/(Positive Control OD-Negative Control OD)].

Descriptive statistics, plots, and tables were prepared using a spreadsheet program (Microsoft Excel). Isotype half-lives from D2 to D7 were calculated using the equation: $T1/2=0.693/k_{elim}$ where k_{elim}=ln(peak concentration)-ln(low concentration)/time interval. Median half-life for each isotype was calculated to prevent undue bias attributable to outliers and is reported in the results.

Data were assessed for homogenous variances and normal distribution using a statistics software package (SAS 9.2). If data was found to have heterogenous variance, log transformation was employed. All transformed data were transformed back to the original scale for presentation in the results. Data groups that were distributed normally were analyzed using ANOVA to determine overall significance and a Dunnett's post hoc test to compare each antibody isotype concentration on D0 to D2, 7, and 14. Data with heterogenous variances that could not be corrected by log transformation was analyzed using the Kruskal-Wallis method to determine overall significance. In these cases, pairwise comparisons between D0 concentrations and D2, 7, and 14 concentrations were made using the Mann-Whitney sign-rank test. Statistical comparisons were classified as significantly different if resulting p-values were less than 0.05.

Results

Average immunoglobulin concentrations in nasal secretions, saliva, and serum are shown by isotype in figure 1. Results of statistical analysis are shown in table 1. Average IgA concentrations in nasal secretions, saliva, and serum increased following colostrum ingestion and were significantly higher than the baseline concentration recorded on D0.

At D7, average IgA concentration declined and was not significantly different than D0 concentration in nasal secretions and saliva. In serum, average IgA concentration declined sharply but remained significantly higher than D0 IgA concentration. On D14, average nasal IgA concentration increased to a level above average serum IgA concentration.

Average IgG1 concentration was significantly higher on D2, 7, and 14 compared to D0 for nasal secretions, saliva, and serum. Average IgG1 concentrations follow a similar pattern over time characterized by a sharp increase following colostrum ingestion at D2 and then steady decline. Overall, IgG1 concentration was very high in serum and much lower in nasal secretions and saliva.

Average IgG2 concentration was significantly higher on D2, 7, and 14 in saliva and serum compared with D0 for saliva and serum, but never for nasal secretions. A very slight increase in IgG2 concentration in saliva and serum can be noted following colostrum ingestion. In contrast to overall serum IgG1 concentration on all days, serum IgG2 concentrations are very low.

Average IgM concentration was significantly higher on D2, 7, and 14 for saliva and serum, and nasal secretions when compared to D0. In contrast to nasal secretion and saliva IgA, IgG1, and IgG2; IgM concentrations rose steadily over the course of the study rather than peaking at D2. Serum IgM concentration followed a similar patter to serum IgG1.

Median half-lives of each isotype are presented in table 2.

BVDV-specific IgA results are presented in figure 2. Average S/P ratio for nasal secretion, saliva, and serum BVDV-specific IgA increased from D0 to D2 following

colostrum ingestion. Nasal BVDV-specific IgA levels plateaued from D2 until D7 and then increased sharply on D14. Nasal BVDV-specific IgA S/P ratios were significantly higher on D2, 7, and 14 compared with D0. Both oral and serum BVDV-specific IgA S/P ratios declined from D2 to D7 and 14. Serum, but not oral, S/P ratios remained significantly different on D7 and 14 compared with D0.

Discussion

Antibodies arrive on mucosal surfaces of adult cattle as the result of either active transport or passive diffusion. IgA and IgM have been shown to reach mucosal surfaces by receptor mediated transfer. This process is characterized by local production of J-chain linked IgA and IgM polymers by plasma cells in the lamina propria of the mucosal surface [7-9], followed by selective transfer of these polymeric antibodies to the mucosal surface by polymeric immunoglobulin receptor [10, 11]. Alternatively, some antibodies reach mucosal surfaces by passive diffusion of serum proteins. [9]. Antibodies of the IgG1 isotype are thought to arrive in the healthy oral cavity as part of a crevicular serum transudate from gingival pockets [12].

The bovine uterine environment prevents exposure and therefor immunologic response to most antigens. This fact coupled with epitheliochorial placentation that prevents transfer of maternal immunoglobulins to fetal circulation results in very low concentrations of antibodies immediately post parturition. Ingestion of antibody-rich colostrum soon after birth leads to absorption of antibodies into the bloodstream. Previous research has indicated that some of these circulating antibodies arrive on mucosal surfaces [4-6].

In this study, transfer of colostral antibodies to circulation was evident. On D0 (precolostral sample), all calves had very low levels of each antibody isotype in serum and by D2, all isotypes had increased significantly. Median half-lives for serum antibody isotypes were comparable to previously published estimates with the notable exception being IgG2 having a median 22.17 day half-life, compared with a previous estimate of 32 days [13]. This finding may be attributable to very lows levels of transfer to serum and loss in mucosal secretions, particularly saliva.

All antibody isotypes were measured at low levels in nasal secretions in the precolostral sample. Following colostrum ingestion, IgA, IgG1, and IgM concentrations all increased significantly by D2. While IgG1and IgM concentrations remained signficanly higher than those found in the precolostral nasal secretion sample, IgA concentrations declined by D7 and then began to increase again on D14, likely as a result of antigenic exposure and response. The mechanism by which IgG1 is transferred to nasal secretions of cattle has not been described, but the IgG1 found on nasal surfaces on D2 can only be attributed to transfer from serum rather than endogenous production following antigenic stimulation. Continuous transfer of serum IgG1 from a robust serum IgG1 pool to nasal secretions coupled with onset of endogenous IgG1 production are likely to account for maintenance of IgG1 concentrations in nasal secretions after D7. Nasal secretion IgM follows a slightly different pattern, characterized by steadily increasing concentrations rather than a peak at D2 followed by a period of decline. IgM is the first antibody isotype that can be detected during an immune response [1]. Peeters et al. demonstrated serum and fecal IgM production in neonatal calves 5 days after exposure to *Cryptosporidium parvum* [14]. In contrast, IgA and IgG production did not

begin to increase significantly until after 7 days post infection. In the case of nasal IgM, concentrations rose significantly above pre-colostral levels by day 2, suggesting the possibility of maternal transfer followed by initiation of endogenous IgM production. The hypothesis that endogenous IgM production begins early is further validated by the fact that IgM levels increased steadily for the duration of the experiment. Antibody concentrations in saliva followed a similar pattern to that seen with nasal antibody, albeit at lower levels.

Generally, median half-lives of nasal secretion antibodies were very short. Mucosal surfaces are protected in part by the flushing action achieved by rapid production and removal of mucosal fluids, e.g. the mucociliary apparatus [15]. This flushing action removes antibodies as well, resulting in a short half-life. The median half-lives of IgM, IgG1, and IgG2 in saliva were all negative in this study. These numbers reflect increasing antibody concentrations over time. Since all half-life calculations were completed based on D2 as the highest concentration and either D7 or 14 as the lowest concentration, a negative median half-life suggests that some animals had increased oral IgM, IgG1, and IgG2 during the calculation interval. In the case of IgM, endogenous production best explains increasing concentrations in this study. However, endogenous IgG1 and IgG2 production is very unlikely by D7 and may be slightly evident by D14. IgG1 in particular has been shown to leak into saliva via capillary leakage from the gingival sulcus [12]. Because of this leakage, continuous leakage of IgG1 into saliva may explain slight increases in concentrations and negative half-lives. Overall low levels of IgG2 make explanation of half-live difficult, but IgG2 may behave in a manner similar to IgG1.

BVDV-specific IgA was detected in nasal secretions, saliva, and serum. In nasal secretions, a small but significant transfer was seen prior to initiation of BVDV-specific IgA production after D7. BVDV-specific IgA production may be the result of exposure to BVDV. The infection status of the other calves co-housed with study calves was unknown. Since nose-to-nose contact was possible, study calves may have been exposed to BVDV very soon after birth. A slight, brief transfer of BVDV-specific IgA was noted in saliva, but the magnitude of transfer was much lower when compared to nasal secretions.

This study demonstrates significant evidence of maternal antibody transfer to the oral and nasal mucosal surfaces of calves. IgA, the most abundant mucosal antibody isotype, demonstrated a clear pattern of transfer that could not reasonably be attributed to any other mechanism due to the time required by a calf to mount an IgA response. Reports have shown that a minimum of 7 days is required to produce a mucosal IgA response to intranasal challenge [6, 16]. These findings taken together with the observation in the present study of significantly increased levels of total IgA by day 2 demonstrated the antibody was almost certainly of maternal origin.

Mucosal fluids are being used as respiratory pathogen diagnostic and research samples in several species [17-19]. Because mucosal antibodies have been shown to be important in both mitigating disease and pathogen surveillance, a thorough understanding of their origin and kinetics is necessary to appropriately apply and interpret mucosal antibody detection assays. Historically, serum antibody responses have been used as a common method of measuring an immune response. These immune responses are used as research outcome variables and as a means of surveying populations for exposure to

disease. Long lasting maternal antibodies confound the use of serum antibody as a tool for these purposes. The work of Brennan, Litt, and Prickett et al shows that mucosal antibodies can be used in research and diagnostic endeavors, but the impact of passively acquired antibodies on mucosal surfaces must be characterized prior to implementing similar techniques in cattle. This research demonstrates that antibody of all isotypes can be measured in the secretions associated with the nasal and oral cavities. There is strong evidence that passively acquired antibody is transferred transiently into mucosal secretions of the mouth and nose. Analysis of pathogen-specific data reveals that mucosal antibody profiles may prove to be a useful tool for detecting acquired immune responses in calves at a much younger age than is currently permitted by serum antibody analysis.

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Tables and figures

Table 1

	IgA			IgG1			IgG2			IgM		
	Nasal	**Oral**	**Serum**	**Nasal**	**Oral**	**Serum**	**Nasal**	**Oral**	**Serum**	**Nasal**	**Oral**	**Serum**
Analysis	**P**	**P**	**N**	**P**	**P**	**P**	**P**	**P**	**N**	**N**	**N**	**N**
D0vsD2	**S**	**S**	**S**	**S**	**S**	**S**	**NS**	**S**	**S**	**S**	**NS**	**S**
D0vsD7	**NS**	**NS**	**S**	**S**	**S**	**S**	**NS**	**S**	**S**	**S**	**S**	**S**
D0vsD14	**S**	**NS**	**S**	**S**	**S**	**S**	**NS**	**S**	**S**	**S**	**S**	**S**

Table 1 shows results of statistical analysis for total Ig class concentration in nasal, oral, and serum samples. Analysis type is represented by P for parametric (Dunnett's post hoc comparisons) and by N for nonparametric (Mann-Whitney pairwise comparisons). S = Significant; NS = Not Significant

Table 2

Isotype	Nasal Secretions	Saliva	Serum
IgA (Range)	2.4 (¯3858.3-5.4)	6.3 (4.3-71.2)	2.9 (2.6-3.2)
IgM (Range)	1.0 (¯16.5-109.6)	-4.1 (¯24.2-¯2.1)	8.6 (6.1-15.3)
IgG1 (Range)	6.6 (¯17.8-17)	-0.7 (¯27.3-29.2)	20.9 (15.1-44.2)
IgG2 (Range)	4.0 (¯47.0-8.6)	-0.2 (¯10.0-19.7)	22.2 (18.1-27.6)

Table 2 shows median half-life (days) and range of half-lives for each antibody isotype in nasal secretions, saliva, and serum.

Figure 1

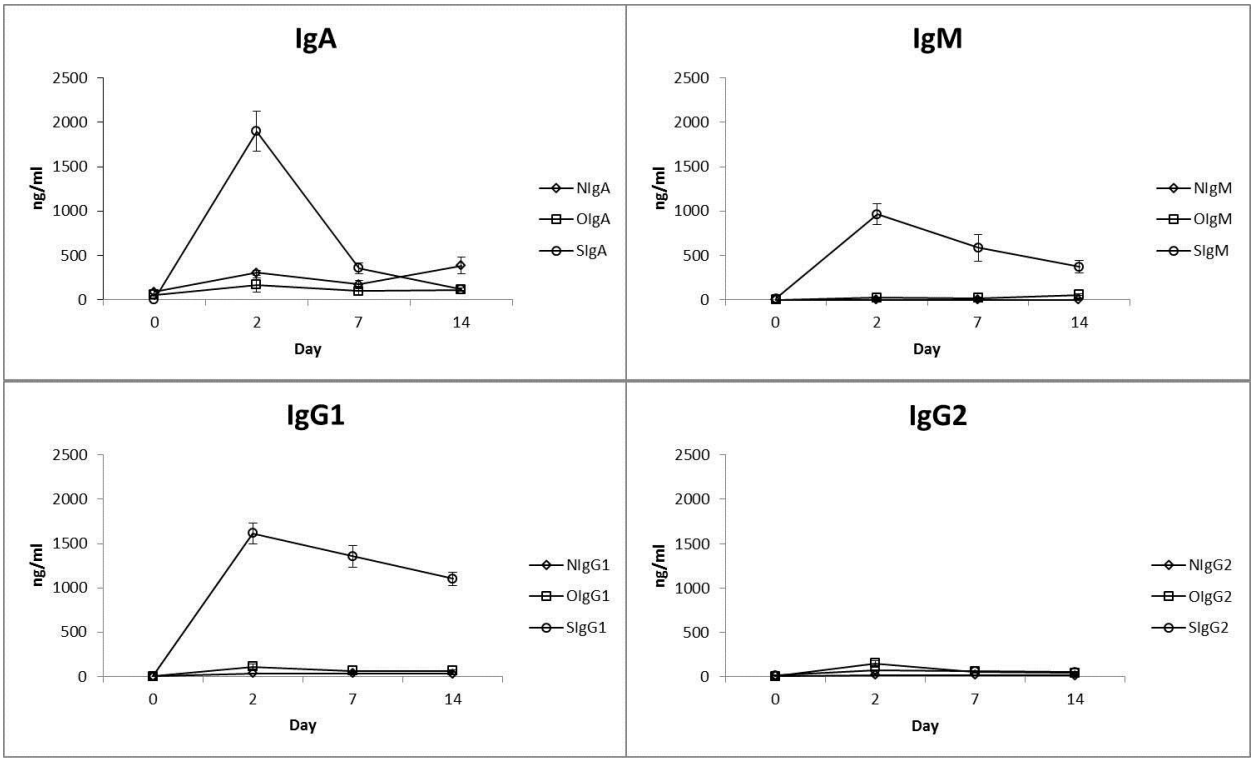

Figure 1. This figure shows the concentrations of each bovine antibody isotype in serum (Sig__), nasal secretions (Nig__), and saliva (Oig__). Results of statistical analysis are presented in table 1.

Figure 2

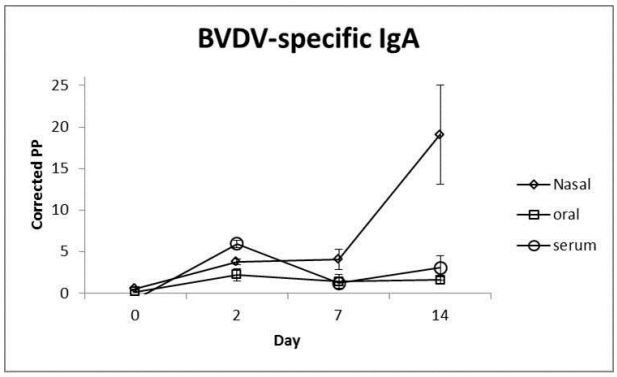

Figure 2. This figure shows BVDV-specific IgA in nasal secretions (nasal), saliva (oral), and serum. Oral IgA concentrations on D7 and D14 are not significantly different from D0, but all other D2, D7, and D14 IgA concentrations are significantly different from D0 measurements.

Chapter 5. Impact of *Bovine viral diarrhea virus-specific* nasal antibody on morbidity and performance of high risk stocker cattle.

A paper to be submitted to *The Journal of Animal Science*

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Abstract

Bovine respiratory disease (BRD) is an economically significant problem impacting all areas of cattle production. The first objective of this research was to measure associations between *Bovine viral diarrhea virus* (BVDV)-specific nasal and serum antibodies, castration, arrival body weight, and arrival rectal temperature with BRD and average daily gain (ADG). A second objective was to measure the effect of vaccination with either modified live virus or killed virus BVDV vaccine on BRD and ADG. Calves were randomly assigned to either MLV or KV groups at enrollment. Intact male cattle were castrated. Rectal temperature and body weight were measured at enrollment. Body weight was measured again on days (D) 14, 28, and 44 of the study. Serum and nasal fluid samples were collected for BVDV-specific antibody analysis on days 0, 14, 28, and 44. Serum and nasal fluid BVDV-specific antibodies were measured using a commercial ELISA. Calves with D0 nasal antibodies (NA) levels below the 50^{th} percentile had 2.26 times the odds of developing BRD compared with calves in the upper 50^{th} percentile ($p<0.0001$). Calves that were castrated on arrival had 2.41 times the odds

of developing BRD compared with animals that were castrated prior to arrival ($p<0.0001$). Calves below the 50^{th} weight percentile had 1.52 times the odds of developing BRD compared to calves above the 50^{th} weight percentile ($p=0.0217$). No significant association between arrival rectal temperature and BRD was discovered in this study. Animals castrated prior to arrival had a 0.52 kg improvement in ADG compared with animals castrated on arrival ($p<0.0001$). A negative linear association was detected between arrival weight and ADG (Slope=-0.002, $p=0.0259$). A moderately significant positive linear association was present between D0 NA and ADG (slope=0.92, $p=0.0852$). The association between MLV or KV and BRD was not significant and was confounded by D0 NA. ADG was 0.15kg ($p=0.0004$) higher for calves in the MLV treated group compared to calves in the KV treated group. Several associations between easily-measured parameters and BRD were established. Some of these, namely castration and weight, have previously been studied. BVDV-specific NA offers a potential mechanism to quantitatively assign newly arrived cattle to risk groups due to its strong association with BRD.

Introduction

Bovine respiratory disease (BRD) is detrimental to the welfare and productivity of cattle. Economic losses incurred due to BRD include treatment costs, mortality, and inefficient growth [1, 2]. Most beef cattle production in the U.S. takes place occurs in two or more production facilities. Usually, cow-calf producers raise newborn calves until weaning age and then move them to forage-based calf development operations (stockers) or to feedlots. BRD risk is highest during and following movement between operations because of exposure to pathogens in marketing facilities and stress related to management

procedures [3]. Management procedures that induce stress, and may reduce resistance to BRD, include weaning, castration for males, dehorning, and shipping. Many producers perform these procedures and vaccinate their calves prior to marketing as part of a practice generally known as preconditioning. Preconditioned calves are physically and immunologically better prepared to remain healthy through transitions to stocker or feedlot operations [3].

Bovine viral diarrhea viruses (BVDV) have been shown as economically significant pathogens in the development of BRD [4]. BVDV is immunosuppressive, acts synergistically with other respiratory pathogens to enhance disease, and can cause primary lung damage [5-7]. Calves that arrive at a feedlot seropositive for BVDV antibodies have been shown to be less likely to develop BRD [8]. Because of these effects, BVDV is a common target of diagnostics and vaccination as part of preconditioning and on arrival at stocker and feedlot operations.

While preconditioning is recognized as a successful BRD prevention strategy, many calves are marketed each year without the benefit of preconditioning. A survey conducted by the United States Department of Agriculture found that 60.6% of surveyed producers never vaccinated cattle prior to marketing (http://www.aphis.usda.gov/animal_health/nahms/beefcowcalf/downloads/beef0708/Beef0708_is_CalfVacc.pdf accessed 10SEP2012). Management information, including information about preconditioning, is not efficiently transferred from seller to buyer in most marketing situations. This communication failure necessitates risk assessment of newly acquired cattle. Because there are few objective tools available for risk assessment, assessment is based on subjective parameters such as appearance, origin, and lot size.

Stocker and feedlot producers compensate for uncertainty in risk assessment by applying broad management strategies that mitigate the impact of BRD. Many stocker and feedlot producers mass medicate cattle determined to be at high risk of respiratory disease with parenteral or oral antimicrobials. Increased production costs and public scrutiny are making these strategies less appealing.

Most cattle are often vaccinated soon after arrival at stocker and feedlot operations to provide protection against respiratory pathogens. Producers implement specific vaccination programs for many reasons including cost, efficacy, and safety. Many viral respiratory vaccines are currently available and generally include a combination of *Bovine herpesevirus-1, Bovine viral diarrhea virus-1, Bovine viral diarrhea virus-2, Parainfluenza virus-3,* and *Bovine respiratory syncytial virus*. Each agent can be present in the vaccine as either modified-live virus (MLV) or inactivated virus (also known as killed virus [KV]).

BRD management would benefit from objective methods of risk assessment that would allow selection of the most efficacious control strategies. Measurement of pathogen-specific nasal antibody offers opportunities to assess immunity against important BRD-related pathogens and thereby provide a direct, quantitative assessment of risk. Reports of association between pathogen-specific nasal antibodies and BRD risk are not available in the current literature. The first objective of this study was to examine the association between arrival BVDV-specific nasal antibodies and BRD. Further, associations of several other variables including arrival BVDV-specific serum antibodies, arrival weight, arrival temperature, and arrival castration status with BRD were

examined. This report does not present a predictive model for BRD; rather it identifies several easily-measured parameters that could be included in a predictive model.

The association of BVDV with BRD has led to widespread vaccination of cattle as they arrive at stocker and feedlot operations. Much debate surrounds the issue of vaccine type administered to high risk cattle. Some producers cite rapid induction of immunity as a reason to use MLV whereas other producers opt for the perceived safety of KV. A second objective of this study is to compare the impact of vaccinating high risk stocker cattle with a vaccine containing KV BVDV compared to a vaccine containing MLV BVDV.

Materials and Methods

The animals in this study were privately owned and managed according to accepted production practices. They were not subjected to any invasive or painful procedures as part of this research. In addition, humane euthanasia protocols were in place to prevent unnecessary animal suffering as a result of naturally occurring disease conditions.

This study was organized as a randomized trial with animal caretakers blinded to experimental groups. Six-hundred and one intact and castrated male, crossbred calves ranging in size from 115 to 287 kg were acquired from a southwestern Missouri auction market. No history could be obtained regarding vaccination or management history of these cattle. Two-hundred ninety-three were enrolled on 15SEP10 and 308 were enrolled on 22SEP10. Prior to enrollment, all animals were rested with hay and water for at least 12 hours after arriving at the ranch. The study location was located in northeastern Oklahoma.

During each enrollment day, calves were randomly assigned to receive either an adjuvanted MLV BVDV respiratory vaccine (Pyramid 5, Boehringer-Ingelheim Vetmedica, St. Joseph, MO) or an adjuvanted KV BVDV respiratory vaccine (Prism 5, Boehringer-Ingelheim Vetmedica, St. Joseph, MO) using prepared randomization schedules. Separate randomization schedules for bulls and steers were utilized to prevent unnecessary bias due to castration stress. In addition to the BVDV component, both vaccines also contained MLV *Bovine herpesvirus 1*, *Parainfluenza virus type 3*, and *Bovine respiratory syncytial virus.* All animals were revaccinated at day 14 of the study with the MLV respiratory vaccine.

Once assigned to a group, each calf received its allocated vaccination, an anthelminthic (Cydectin Injectable, Boehringer-Ingelheim Vetmedica, St. Joseph, MO), metaphylaxis (Excede, Pfizer Animal Health, Kalamazoo, MI), a *Mannheimia haemolytica* vaccine (Presponse SQ, Boehringer-Ingelheim Vetmedica, St. Joseph, MO), an 8-way clostridial vaccine (Covexin 8, Merck Animal Health, Summit, NJ), and two unique ear tags. Bulls were castrated via banding with the exception of sixteen bulls that required surgical castration due to poorly positioned testicles. Fifty-three calves had horns that were blunted by removing 2-3 cm of each horn (tipping). Ear notch samples were collected for BVDV PI testing (IDEXX Herdchek BVDV-Ag ELISA, Westbrook, ME). All events described were included in the study record for the appropriate group along with a description of each calf, initial rectal temperature, and initial weight.

Animals with existing disease conditions at the time of enrollment were excluded from the study. Disease conditions included respiratory disease, lameness, bloat, or other

debilitating conditions that could bias to a particular research group. BVDV PI animals were removed within 24 hours of induction.

Throughout the trial health events were recorded. BRD was defined as any animal with a clinical illness score (CIS) $\geq$1 and a rectal temperature $\geq$104.0°F. CIS was defined as 0 for animals that did not appear ill, 1 for animals that appeared mildly ill, 2 for animals that were moderately ill, 3 for animals that were severely ill, and 4 for animals that were moribund. This scoring system was based on subjective assessment of animal status by the animal caretakers. Animals were given Baytril (Bayer Animal Health, Shawnee Mission, Kansas) for their first BRD event, Nuflor (Merck Animal Health, Summit, NJ) for their second BRD event, and Biomycin (Boehringer-Ingelheim Vetmedica, St. Joseph, MO) for their third event after which they were no longer eligible for treatment. At the time of each event, date, rectal temperature, and weight were recorded. All mortalities were necropsied and the results were recorded in the treatment record.

Calves were housed on grass pastures and supplemented with a pelletized protein feed. See Table 1for ingredients and analysis.

Nasal swabs and blood were collected from each calf at enrollment (D0) and on days 14, 28, and 44. Nasal swabs were collected by rotating a nylon flocked swab (Puritan Medical Products, Guilford, ME) in one or both nasal passages of each calf until the swab was saturated. The swab was then placed in a 1.5 ml microcentrifuge tube labeled with the calf's ID. Blood was collected via caudal vein venapuncture using Vacutainer needles and 10 mL serum separator tubes (BD, Franklin Lakes, NJ). After collection, the tube was labeled with the calf's ID. All samples were placed on ice for

transport to the laboratory. Nasal swabs were frozen for long term storage. Blood tubes were centrifuged at 1000 x *g* for 20 minutes at 22ºC and the serum was decanted into 5 ml serum storage tubes and refrigerated.

Nasal swab samples were processed by thawing and then adding 300 µl PBS with 1 mM EDTA followed by 400 µl of prepared dithiothreitol (DTT) reagent (Sputolysin Reagent, EMD Millipore, Rockland, MA). The nasal samples, including the swab, were vortexed and incubated at room temperature (22ºC) for 15 minutes. After the incubation, the samples were centrifuged for 3 minutes at 3000 x *g* and supernatant removed for analysis.

Nasal and serum samples were assayed for the presence of BVDV-specific antibodies by means of a commercially available ELISA kit (Svanova BVDV-Ab, Uppsala, Sweden). Nasal antibody detection was achieved by spiking the conjugate provided in the kit with 2 ul/plate (1:5,000 dilution) sheep anti-bovine IgA antibody (Bethyl Laboratories, Montgomery TX). Kit protocol was followed with the notable exceptions that positive and negative control serum were diluted 5-fold in PBS for use as controls in the nasal antibody assay and incubation time was extended from 10 minutes to 20 minutes during the substrate color development step. Intra-assay and interassay coefficients of variations (CV) were calculated from sample to positive (S/P) ratio data for nasal antibody measurements and from percent positivity measurements for serum antibody measurements. The following equations were used to standardize ELISA results.

BVDV specific nasal antibody S/P = (Sample OD-Negative control OD)/(Positive control OD-Negative control OD).

BVDV specific serum antibody PP = (Sample OD/Positive control OD)*100

Descriptive analysis of variables and treatment success data were performed using Microsoft Excel. Categorical variables collected include respiratory disease (yes or no), treatment group (MLV or KV), castration status (bull or steer), dehorn (yes or no), and enrollment set (1 or 2). Continuous variables collected included arrival temperature, arrival weight, D14 weight, D28 weight, D44 weight, D0 nasal antibody (NA), D14 NA, D28 NA, D44 NA, D0 serum antibody (SA), D14 SA, D28 SA, and D44 SA. Average daily gain was calculated for each calf by subtracting D0 weight from D44 weight and dividing by 44. Average daily gain was calculated for all animals, including mortalities. Treatment success variables calculated from treatment records included treatment success risk, treatment failure risk, relapse risk, second relapse risk, and new episode risk. See Table 2 for category definitions.

Statistical analyses were performed using SAS 9.2 (SAS Institute, Cary, NC), specifically the mixed models procedure and logistic regression procedure. All continuous variables were either categorized into biologically relevant groups (i.e. hyperthermia and normothermia for temperature data) or were categorized into high and low observations corresponding to the upper and lower 50^{th} percentiles of observations. Morbidity data was analyzed using logistic regression with respiratory disease as the outcome variable of interest. For each model built, only one explanatory variable was designated as the explanatory variable of interest (i.e. a model for association of vaccination strategy and respiratory disease and a completely separate model for association of NA and respiratory disease). A univariate model containing only respiratory disease and the explanatory variable of interest was created to obtain an

unadjusted odds ratio (OR), then all relevant variables were added to create a full model. Interaction terms for enrollment set were evaluated at this stage. In the case of significant enrollment set interaction, a separate model for each enrollment set was generated. After an appropriate full model was built, variables with Wald type III p-values greater than 0.1 were removed stepwise starting with the largest p-value. After each variable was removed, the OR for the explanatory variable of interest was assessed for significant change (>20%). If the change was greater than 20% when compared to the full model, the variable was forced back into the model as a confounding variable regardless of significance.

Mixed models procedures were used to compare NA and SA terms at each time point. Each time point was analyzed for assumptions. In the case of non-normal distributions, observations were transformed by setting the lowest observation equal to 0 and then adding the value needed to raise the lowest observation to 0 to all other observations. Adjusted observations were then natural log transformed . Estimates of means were back transformed and are presented in the results in their original form. In each analysis, vaccination group was the explanatory variable and enrollment set was included as a random variable to account for its influence on the relationship between vaccination and the measured/calculated variables.

Results

Of the 601 animals enrolled in the study, 583 animals with complete data sets were included in the final data analysis. Two animals were removed from the study at the time of enrollment, one due to recurrent bloat and the other due to existing chronic respiratory disease. Four animals, 1 from each pen of calves, were removed after being

diagnosed as a BVDV PI. Randomization successfully allocated 1 PI calf in each of the 4 groups of calves, affording equal BVDV exposure opportunity across all groups. Four animals died during the study. Observations from mortalities were included in all applicable analysis, including ADG. Twelve animals were randomly selected for assay optimization; therefore, the observations from these animals were not included in the statistical analysis. Of the animals included in the statistical analysis, 53.7% were bulls, of which 16 required surgical castration. Fifty-three calves were dehorned. See Table 3 for a summary of BRD by enrollment group.

Intra-assay and interassay CV values for the nasal antibody ELISA were 2.3% and 2.6%, respectively. Intra-assay and interassay CV values for the serum antibody ELISA were 2.8% and 8.5%, respectively.

Results of analysis of factors significantly associated with respiratory disease are presented in Table 4. Confounding analysis of the relationship between BRD and vaccine type by D0 nasal antibody revealed a change in OR of greater than 20%. Results of factors significantly associated with ADG are presented in Table 5. A plot of ADG by treatment group is shown in Figure 1.

Discussion

Serologic immunity against many respiratory pathogens, including BVDV, has been associated with decreased BRD morbidity in calves entering feedlot operations [8]. In field conditions, animals will have variable immune protection against BRD and information regarding vaccination or prior infection is often unavailable. This lack of information coupled with subjective risk assessment methods makes discernment of BRD risk in groups of cattle difficult.

Several reports of risk factors associated with BRD have been reported. Cernicchiaro measured associations of shrink, travel distance to the feedlot, and weather conditions with BRD [9-11]. Others have attempted to use biomarkers to predict BRD [12]. An inflammatory biomarker, haptoglobin, was shown to be unsuccessful as a predictor of BRD risk [13]. The etiology of BRD is complex and involves interactions of stress, pathogen and host immune response. Current predictive models lack easily-measured variables related to immunologic protection against respiratory pathogens. At least one report has been published regarding the association between SA specific to several BRD pathogens and the incidence of BRD in the feedlot [8]. However, SA may not be an ideal representation of immune status due to the presence of waning colostral antibody [14, 15]. Since SA confounds detection of active immunity, both SA and NA levels were evaluated for potential impact on both BRD morbidity and ADG. Arrival SA was not found to be significantly associated with odds of BRD in this study. In contrast, high arrival NA levels specific to BVDV were associated with decreased odds of developing respiratory disease during the study. Similarly, serum antibody was not associated with ADG whereas arrival NA tended to be linearly related to ADG in a positive fashion.

Previous research has shown that maternal antibody can be transferred to the nasal secretions of neonatal calves [16]. These antibodies usually persist for less than 3 weeks [17]. Pathogen-specific nasal antibodies found after 3 weeks of age can be attributed to endogenous active immune responses. Demonstration of a relationship between arrival NA specific to BVDV, a known contributor to BRD, and reduced respiratory disease reveals the impact of a complete, active immune response. In contrast, SA does not

appear to be as closely tied to BRD risk. Serum antibodies in calves of this age are present either as a result of maternal transfer or as a result of an endogenous, active immune response. This result may reflect the imperfect protection afforded by antibodies alone as opposed to the more complete immune response necessary to produce NA. The findings related to BVDV-specific NA on arrival confirm the association of active immunity with decreased odds of BRD. One explanation for the beneficial associations of BVDV-specific NA would be that an actively acquired immune response results in a more rapid control of BVDV infection. Previous research showed that calves vaccinated against BVDV and then challenged with highly virulent BVDV did not become viremic, had a reduced febrile response following challenge, and experienced less severe clinical disease compared to calves with no previous exposure to BVDV [18]. In the present study, BVDV-specific NA would be present as a result of a previous immune response. This response would result in both humoral and cell mediated immune defense mechanisms directed against a BVDV challenge [19].

Vaccination against respiratory pathogens, including BVDV, is a common whole-herd practice used by cattle producers to reduce respiratory disease losses. Producers can choose from a variety of vaccine types that include MLV, KV, and combinations of the two. MLV BVDV vaccines have been shown to cause changes in immune cell function, leading to concerns about immune suppression when combined with weaning and shipping stress [20]. While KV vaccines are thought to have fewer adverse effects, KV vaccination does not typically result in induction of cytotoxic T-lymphocytes [21]. These lymphocytes are stimulated following vaccination with MLV and are thought to be important for controlling BVDV infections [22]. In the present study, impact of vaccine

type on BRD was inconsistent between enrollment. Further statistical analysis showed that the association between vaccine type and BRD morbidity was confounded by D0 NA. D0 NA satisfies the *a priori* criteria used to define a confounding variable [23].

Producers are concerned about the production losses resulting from morbidity and mortality associated with BRD. Vaccination with a MLV was associated with higher ADG than was vaccination with KV. The difference in ADG may be attributable to better protection associated with MLV vaccination. Conversely, the difference in ADG may be associated with increased inflammation associated with the heavier antigen load in the KV vaccine.

Overall, the significant association of BRD morbidity and NA specific to BVDV suggests a new metric that could be used in risk assessment of stocker cattle. More research needs to be done to validate the relationship between mucosal antibody and BRD.

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Tables and figures

Table 1

Pellet Ingredient	% of Formulation
Wheat midds	25
Corn gluten	25
Soybean hulls	25
Corn distiller's grain	12.5
Rice hulls	2.5
Limestone	2.5
Molasses	7.5
Analysis	
Nutrient	Composition (Dry Matter Basis)
NEm MCal/CWT	77.41
NEg MCal/CWT	60.43
TDN %	73.34
Crude protein %	14.69
Fat %	3.73
Crude fiber %	16.24
ADF	22.43
NDF	51.22
eNDF	19.69

Table 2

Therapy Response Variable	Description
Treatment Success	An animal that is fully recovered following initial antibiotic therapy period, no additional therapy required within 21 days of initial therapy.
Treatment Failure	An animal that at 72 hours post initial therapy, clinical illness score (CIS*) is greater than time 0 CIS or CIS is 1 or greater and rectal temperature is $\geq$104.0° F.
Relapse	An animal that is deemed recovered at 72 hours post initial therapy but is observed with signs of BRD (CIS $\geq$ 1) and has a rectal temperature $\geq$ 104° F and is $\leq$ 21 days from the initial therapy.
Second Relapse	An animal that contracts BRD within 21 days of second therapy.
New Episode	An animal that is observed with signs of BRD (CIS $\geq$ 1) and has a rectal temperature $\geq$ 104° F and necessitates treatment $\geq$ 21 days following the previous therapy.

Table 2 summarizes the definitions of therapy response variables.

* CIS refers to clinical illness score which is a scale of 0-4 where 0 indicates no apparent illness, 1 indicates mild illness, 2 indicates moderate illness, 3 indicates severe illness, and 4 indicates a moribund state. CIS is a subjective measure of illness based on animal caretakers' experience.

Table 3 Summary of health events by treatment group

	MLV BVDV	KV BVDV	P
Group Size	294	287	ND
New BRD Events	98	102	0.7404
BRD Relapses	37	43	0.4017
Second BRD Relapse	10	10	0.9563
BVDV PIs Removed	2	2	ND
BRD Mortalities	2	1	ND

Table 3 summarizes health events according to treatment group.

Table 4 Factors significantly associated with new BRD events

Variable	Odds Ratio Estimate	Lower 95% Confidence Limit	Upper 95% Confidence Limit	P-value
Vaccine type divided by Enrollment group				
Enrollment Group1				
MLV	Referent			
KV	1.89	1.11	3.18	0.0180
Enrollment Group 2				
MLV	Referent			
KV	0.55	0.33	0.93	0.0265
Overall Analysis				
D0 Nasal Ab				
Low	2.26	1.57	3.25	<0.0001
High	Referent			
Castration Status				
Steer	Referent			
Bull	2.41	1.68	3.47	<0.0001
Weight				
$<50^{th}$ Percentile	1.52	1.06	2.18	0.0217
$>50^{th}$ Percentile	Referent			

Table 5 Factors significantly associated with ADG

Factor	Estimate	Lower 95% Confidence Limit	Upper 95% Confidence Limit	P-value
MLV-KV*	0.15 kg	0.067 kg	0.23 kg	0.0004
Steer-Bull*	0.5187	0.3423	0.6952	<0.0001
D0 Nasal Ab	0.92§	-0.13	1.97	0.0852
Weight	-0.002§	-0.004	-0.0002	0.0259

*Indicates the difference between two groups (i.e. KV group ADG subtracted from MLV group ADG.

§Slope estimates for the linear relationship between the explanatory variable and ADG.

Figure 1

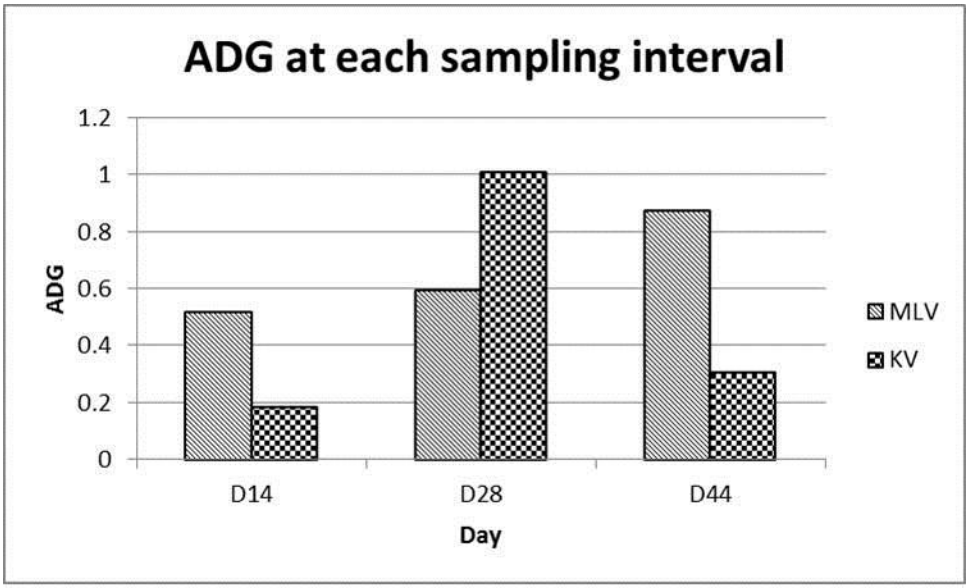

Figure 1 shows the ADG by vaccine type for each sampling interval (e.g. D14 represents ADG between D0 and D14, D28 represents the ADG between D14 and D28, and so on).

Chapter 6. Conclusions

Contributions to bovine viral diarrhea virus knowledge

The first objective for the research presented in this dissertation focused on evaluating alternative sample types for BVDV detection. Currently, a skin biopsy from the ear is the most common sample type. Other sample types that were evaluated include caudal tail fold biopsy, serum, nasal swab, conjunctival swab, vaginal preputial swab, oral swab, and rectal swab. When compared with ear notch ELISA, only caudal tail fold biopsy and nasal swabs correctly identified each persistently infected BVDV animal. Each of the other sample types incorrectly categorized positive animals as negative. This work is the first to describe a comprehensive evaluation of several sample types from known BVDV PI calves. The study did not include negative animals and therefore cannot predict the success of correctly categorizing negative animals with each sample type. Nasal swabs and caudal tail fold skin biopsies were shown to be accurate alternative samples for the identification of BVDV PI calves with the E^{rns} ELISA.

The second objective of this dissertation was to evaluate a common assumption that all haired bovine skin can be used as a diagnostic specimen for antigen based ELISAs. Two commercially available antigen capture ELISAs were evaluated a study to address this objective. The entire hide from 3 BVDV PI cattle was removed, subsectioned, subsampled, and assayed using an ELISA that detected the E^{rns} protein of BVDV and an ELISA that detected the NS2/3 protein of BVDV. This project demonstrated that the E^{rns} ELISA was able to identify all subsections of the 3 animals as positive, but the NS2/3 ELISA was only able to identify a portion of the subsections as positive. Overall, this study highlighted the individual performance of the two evaluated

assays. More importantly, this study demonstrated the need to validate samples prior before using them as positive controls, negative controls, or diagnostic samples for BVDV detection assays.

The third objective of this dissertation was to quantify the transfer and duration of maternal antibodies to the mucosal surface of neonatal calves. This objective was addressed by capturing pre-colostral and serial post-colostral saliva and nasal secretion samples. Total immunoglobulin isotype concentrations and BVDV-specific nasal IgA levels were measured from each of these samples. Overall, a clear, transient transfer of colostral antibody could be detected. This is critical information to future development of mucosal antibody diagnostic techniques. Currently, serum antibody diagnostic techniques are confounded by the presence of maternal antibody in young calves. A clear understanding of the kinetics of mucosal antibodies will enable development of diagnostic techniques that are not confounded by the presence of maternal antibodies.

The fourth objective was to determine the impact of BVDV specific mucosal antibody in calves exposed to BVDV in the field. The study presented in chapter 5 addressed this objective by measuring BVDV specific nasal antibody in calves exposed to BVDV PI cattle. The animals enrolled in this study were stressed, highly commingled, auction market sourced animals that were at high risk of developing respiratory disease. Animals that had high levels of BVDV specific antibody were found to be at lower risk of developing respiratory disease compared to animals that had low levels of BVDV specific antibody. This finding is important because it demonstrates the importance of protection from BVDV in cattle that are at high risk of developing respiratory disease as

well as illustrating the potential damage of encountering BVDV without established protection.

Future Research

Mucosal antibody responses to BVDV and many other respiratory pathogens have not been well characterized. The mucosal antibody response of different vaccine types and routes has been investigated for BRSV [1], but not for BVDV or other respiratory pathogens. Research in this area is important because prior research has shown that BRSV does not illicit the same response following its use in parenteral vaccines when compared with other common components of respiratory vaccines [2-4].

BVDV surveillance using antibody responses is not routinely used in the United States because of the prevalence of antibody response attributable to maternal antibody or vaccination. Controlled studies detailing the dynamics of BVDV mucosal antibody responses may offer potential surveillance techniques that are not affected by maternal antibody or vaccination. Research in other species has already demonstrated the value of mucosal antibodies for surveillance [5].

Bovine respiratory disease risk assessment is based on subjective information. The research presented in this dissertation shows a significant relationship between BVDV specific antibody and risk of respiratory disease in high risk stocker cattle. This information, if properly validated, could serve as an important step in developing quantitative risk assessment models for respiratory disease. More information about the kinetics of mucosal antibody responses to BVDV and other respiratory pathogens as well as information about other risk factors must be evaluated.

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