Evaluation of passive immunity and different vaccination protocols on the induction of local and systemic antibody responses and clinical protection of beef calves against experimental infection with bovine respiratory syncytial virus (BRSV)

by

David Alexander Martínez Rodríguez

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Approved by

Manuel F. Chamorro, Chair, Associate Professor of Clinical Sciences
Amelia Woolums, Professor of Pathobiology and Population Medicine
Thomas Passler, Professor of Clinical Sciences
Paul H. Walz, Professor and Department Head of Pathobiology
Ricardo Stockler, Director of Elba Regional Veterinary Diagnostic Laboratory
Shollie Falkenberg, Associate Professor of Pathobiology

Abstract

Bovine respiratory syncytial virus (BRSV) is one of the most prevalent viruses associated with the bovine respiratory disease complex (BRDC). The persistency over time and role of local and systemic antibodies against BRSV transferred from maternal colostrum is not completely understood. Additionally, the efficacy of vaccination of calves on providing clinical protection against experimental infection with BRSV or natural occurrence of BRDC is inconsistent in the literature. The objective of this research was to review the literature on the effect of BRSV vaccination, evaluate the effect of local and systemic BRSV antibodies derived from colostrum, and evaluate the effect of different vaccination protocols with MLV BRSV vaccines on the induction of local and systemic antibody responses and clinical protection of beef calves against experimental viral infection.

Results from the meta-analysis indicated that commercially available MLV BRSV vaccines reduced the risk of calf mortality after experimental infection with BRSV. Modified-live virus vaccines reduced the risk of morbidity following experimental infection in calves with absence of serum BRSV antibodies at initial vaccination, but failed to demonstrate significant morbidity reduction when calves were vaccinated in the face of maternal antibodies (IFOMA).

The results from these studies suggest that the presence of colostrum-derived SN antibody titers and BRSV IgG1 transferred to the upper respiratory tract of newborn calves likely plays a role in clinical protection against clinical disease (morbidity) early in life; however, the presence of systemic and local colostrum-derived antibodies interfere with the induction of adequate/complete immune responses to IN MLV vaccination during the first month of life.

Modified-live virus IN vaccination of neonatal calves during the first hours of life and before complete transfer of specific BRSV antibodies from colostrum did not result in priming of nasal BRSV IgA responses following vaccination. Additionally, IN MLV vaccination of neonatal calves did not result in significantly different upper respiratory tract BRSV IgA titers following exposure to BRSV later in life when compared with control calves.

A combination SC-IN MLV vaccination protocol at branding and at weaning, respectively, or IN MLV vaccination at weaning alone did not provide clinical advantages nor resulted in SN antibody response differences compared with no vaccination following challenge of calves shortly after IN vaccination. The upper respiratory tract BRDC-associated bacteria were altered by either IN MLV BRSV and BHV-1 MLV, simultaneous experimental challenge with BRSV and BHV-1 or both in weaning age beef steers.

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List of abbreviations

BRDC	Bovine respiratory disease complex
BHV1	Bovine herpesvirus 1
IBR	Bovine rhinotracheitis
IPV	Infectious pustular vulvovaginitis
IPB	Infectious pustular balanopostitis
BVDV	Bovine Viral Diarrhea Virus
PI	Persistent infection
BCoV	Bovine Coronavirus
CD	Calf Diarrhea
WD	Winter Dysentery
PI3	Bovine Parainfluenza-3 virus
BRSV	Bovine Respiratory Syncytial Virus
LPS	Lipopolysaccharide
G	Attachment glycoprotein
F	Fusion protein
SH	Small hydrophobic protein
M	The matrix protein
N	The nucleoprotein
P	Phosphoprotein
L	RNA polymerase
NS	Non-structural proteins
IL	Interleukins

IFN	Interferon
TNFα	Tumor necrosis factor-α
RANTES	Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted
MCP-1	Monocyte chemoattractant Protein-1
MIP-1α	Macrophage inflammatory protein-1 alpha
RS	Respiratory Syncytial Virus
TLRs	Toll-like receptors
DC	Dendritic cell
APC	Antigen presenting cell
ALDC	Afferent lymph dendritic cells
BAL	Bronchoalveolar lavage
IgM	Immunoglobulin M
IgA	Immunoglobulin A
IgG	Immunoglobulin G
ELISA	Enzyme-linked immunosorbent assay
SN	Serum neutralization
NS	Nasal secretions
NPS	Nasopharyngeal swabs
TTW	Transtracheal wash
PCR	Polymerase chain reaction
IF	Immunofluorescence
IHC	Immunohistochemistry
IFA	Immunofluorescent antibody

d-FAT	Direct fluorescent antibody test
NSAIDs	non-steroidal anti-inflammatory drugs
IFOMA	In the face of maternal antibodies
MLV	Modified-live vaccines
IN	Intranasal
RR	Risk ratio
MDBK	Marvin Darby bovine kidney
RT	Reverse transcription
BW	Body weights
ADG	Average daily gain
IACUC	Institutional Animal Care approved the study and Use Committee
SNA	Serum neutralizing antibody
CT	Cycle threshold
IQR	Interquartile range

Chapter 1: Introduction

The bovine respiratory disease complex (BRDC) is one of the most expensive diseases in the cattle industry in the United States (U.S.); economic losses are associated with mortality, morbidity/treatment, decreased weight gain, labor, and time required for treatment.(1, 2) This disease (BRDC) results from a combination of risk factors and different pathogenic agents that include viruses and bacteria.(3) Bovine respiratory syncytial virus is one of the most prevalent viruses in cattle populations in the United States, and it has been associated with BRDC outbreaks in dairy and beef calves by itself or through synergistic infections with other viruses and bacteria.(4) Cattle become susceptible to BRSV infection when maternal antibodies decay, in cases of failure in the transfer of passive immunoglobulins from colostrum, or following exposure to stressful or immunosuppressive factors such as branding, weaning, transportation, comingling, or general management procedures (i.e., castration, dehorning, etc.).(4) Different efforts have been done to reduce calf morbidity and mortality associated with BRSV infection and disease in calves. Within these efforts, vaccination at different calf ages has become the most commonly adopted strategy by producers and veterinarians; however, results from different clinical trials including experimental BRSV infection and natural occurrence of BRDC are inconsistent regarding the efficacy of vaccination on clinical protection.(5-9) The studies included in this dissertation address some of the lack of knowledge in relation to vaccination protocols in cow-calf herds that may increase the transfer of specific passive immunity to young calves and/or induce adequate local and systemic BRSV antibody responses that result in clinical protection of calves during the pre-weaning period and early post-weaning against experimental infection with BRSV.

Chapter 2 describes the current state of the art and knowledge related to BRDC with specific focus on beef industry. This chapter describes the epidemiology and the most important risk factors associated with the clinical presentation of BRDC including weaning, transportation, environmental conditions, source of the animals before arrival to the feedlot, stressors such as dehorning and castration, animal factors such as age, weight and gender, genetics, and the role of local microbiome in the upper and lower respiratory tract. A summary of the most important infectious agents including the most prevalent viruses and bacteria as well as the economic impact of the BRDC in the U.S. beef industry are included in this chapter. Finally, a thorough literature review of BRSV focusing on the pathophysiological mechanisms of the virus and interaction with the immune system, BRSV epidemiology including mechanisms of transmission, clinical signs associated, and different forms of presentation, available diagnostic tests, treatment and prevention strategies have been included.

Chapter 3 describes the research objectives of the present dissertation. First to perform a systematic review of literature on the efficacy of the commercially available vaccines against BRSV experimental infection in calves. Second, to evaluate the effect of vaccination of dams during gestation with a KV BRSV vaccine on the transfer of passive immunity and subsequent clinical protection of calves against experimental BRSV infection. Third, to evaluate the effect IN MLV vaccination of neonatal calves before complete absorption of passive antibodies from colostrum on BRSV antibody responses and clinical protection of calves against experimental BRSV infection. Fourth, to evaluate the effect of vaccination of beef steers with a combination vaccine protocol at branding age (2 months of age) and weaning (6-8 months of age) with MLV

subcutaneous (SC) and MLV IN BRSV vaccines, respectively, versus single IN MLV BRSV vaccination at weaning on SN and nasal IgA antibody responses and clinical protection against simultaneous challenge with BRSV and bovine herpesvirus 1 (BHV-1) 2 days following IN vaccination.

Chapter 4 is a systematic review and meta-analysis evaluating available literature on the efficacy of MLV and KV BRSV vaccines to provide clinical protection against experimental BRSV infection in young calves. This meta-analysis indicated that commercially available MLV BRSV vaccines reduce the risk of calf mortality after experimental infection with BRSV. Modified-live virus vaccines reduce the risk of morbidity following experimental infection in calves with absence of serum BRSV antibodies at initial vaccination, but fail to demonstrate significant morbidity reduction when calves are vaccinated in the face of maternal antibodies (IFOMA). The number of studies that evaluated KV BRSV vaccines within the meta-analysis was insufficient to provide significant conclusions on the effect of vaccination on calf morbidity and mortality.

Chapter 5 describes a randomized clinical trial that evaluated initial titers, duration, and clinical protection of passively-transferred BRSV nasal immunoglobulin G-1 (IgG1) and immunoglobulin A (IgA), and serum neutralizing (SN) antibodies in beef calves born to dams vaccinated or not during the last trimester of gestation with a KV BRSV vaccine, with the calves subsequently challenged with BRSV at approximately 3 months of age. Signs of respiratory disease after challenge were similar between groups. Nasal BRSV IgG1 and SN antibodies were significantly greater in calves born to vaccinated dams at 48 hours of life; however, by 3 months

of age, titers had decayed in both groups and were not significantly different. Nasal BRSV IgA titers were almost undetectable before BRSV challenge and increased in both groups after challenge. The probability of shedding BRSV following experimental challenge was significantly greater in the group of calves born to unvaccinated dams.

Chapter 6 describes a randomized clinical trial that evaluated the effect of vaccination of beef calves within the first 6 hours of birth (before complete absorption of colostral immunoglobulins) with a single dose of an intranasal (IN) MLV BRSV vaccine on local and systemic antibody responses and clinical protection against experimental BRSV challenge at approximately 3.5 months of age. The control group received intranasal saline in lieu of vaccination. Signs of respiratory disease were not significantly different between vaccinated and unvaccinated groups following BRSV challenge; however, on challenge day, >40% of calves in each group presented with fever (rectal temperature > 39.7°C). The SN and nasal IgA BRSV antibody responses before challenge (at 1 month of age and on challenge day) suggested natural exposure to BRSV prior to challenge. All animals tested positive for BRSV in nasal secretion samples following challenge and nasal shedding based on quantitative (q) RT-PCR was not significantly different between groups.

Chapter 7 describes a randomized clinical trial that evaluated the effect of vaccination of beef steers with a combination vaccine protocol at branding age (2 months of age) and weaning (6-8 months of age) with MLV subcutaneous (SC) and MLV IN BRSV vaccines, respectively, versus single IN MLV BRSV vaccination at weaning on SN and nasal IgA antibody responses and clinical protection against simultaneous challenge with BRSV and bovine herpesvirus 1

(BHV-1) 2 days following IN vaccination. A non-vaccinated control group was included. Signs of respiratory disease were not significantly different between treatment groups following BRSV and BHV-1 challenge. Regardless of treatment group, based on q-PCR testing a greater proportion of calves shed BHV-1 in nasal secretions compared with BRSV following IN MLV vaccination and challenge. The mean log2 SN antibody titers to BRSV and BHV-1 before and after challenge were not significantly different between treatment groups. All calves were negative to *Histophilus somni* and *Mycoplasma bovis* q-PCR in nasal secretion samples before IN vaccination and following vaccination and experimental challenge. *Mannheimia haemolytica* and *Pasterella multocida* were detected in a moderate proportion of nasal secretion samples before vaccination and challenge; however, their detection significantly increased and peaked by Day 7 post-challenge in all calves. Differences in the presence of bacteria in nasal secretion samples between treatment groups were not statistically significant.

Chapter 8 includes the conclusions of the results from the studies performed within this research program and potential directions of future research based on them.

Chapter 2: Literature review

Bovine Respiratory Disease Complex

The bovine respiratory disease complex (BRDC) was initially defined as a pathologic condition composed of three different syndromes including enzootic calf pneumonia, shipping fever, and atypical interstitial pneumonia;(3) however, it is accepted that bronchopneumonia or pleuropneumonia resulting from BRDC in cattle is usually the consequence of interaction of different viral and bacterial pathogens, compromised immune responses following weaning, transportation and commingling, and dietary and environmental factors. An initial viral respiratory infection and consequent compromise of the upper respiratory tract defense mechanisms and immune system sets up the conditions for a secondary bacterial bronchopneumonia.(10) In most cases of BRDC, respiratory viruses and/or bacteria are detected in the lower respiratory tract of affected animals.(3) Cattle with clinical BRDC can develop different clinical signs, including increased body temperature, decreased feed intake, altered mentation, decreased milk production, nasal discharge, cough, altered respiratory dynamics or dyspnea, increased respiratory rate, ocular discharge or lacrimation, and abnormal lung sounds among others.(11) Bovine respiratory disease is one the most important causes of economic loss to the beef industry in the United States and worldwide and these losses are associated with mortality, treatment administration, reduced performance, preventive measures, and labor.(12-14)

Epidemiology

Bovine respiratory disease is one of the most studied conditions in cattle production. And a 9-year-review of the disease in feedlot operations between 1986 and 1994 demonstrated a 7.8% morbidity and 0.86% mortality.(15) More recently, BRDC has been associated with 60 to 90% of feedlot cattle morbidity and mortality in feedlot cattle in the United States.(16) Additionally, an increasing prevalence of BRDC over time has been reported in U.S. feedlots.(17, 18) Official statistics from the USDA indicate that in 1999 the percent of cattle affected by BRDC in U.S. feedlots was 14.4%; however, this value increased to 16.2% in 2001.(12). Results from one study demonstrated an increase in BRDC-associated mortality in U.S. feedlots from 10.3 deaths/1000 animals in 1994 to 14.2 deaths/1000 animals in 1999, with new cattle entering the feedlot in 1999 having a greater risk of BRDC compared with the same group in 1994.(19) The bovine respiratory disease complex is considered a multifactorial syndrome in which infectious agents, management, and environment play a role on its clinical presentation. Some of the predisposing factors for BRDC include weaning, transportation, commingling, dust, weather changes, dehydration, exposure to endotoxins, cold temperatures and humidity, and metabolic disturbances.(3, 20)

Predisposing factors

Weaning

By definition, weaning is the abrupt separation of calves from dams with complete interruption of milk feeding. In the majority of beef cow-calf U.S. operations, weaning of calves occurs between 6 and 9 months of age and usually involves abrupt separation from dams and immediate shipping.(21) In the majority of U.S. dairy operations, calves are separated from their dam immediately after birth, and whole milk or milk replacer feeding is stopped between 6-8 weeks of age.(22) The stress of weaning leads to physiological responses such as increases in plasma cortisol, norepinephrine, and acute phase proteins, and depending on their duration can negatively impact the immune system and predispose the calf to infectious disease such as BRDC.(23, 24) The stress associated with separation from the dam in beef calves is usually intensified by other changes around the time or immediately following weaning including castration, transport, commingling with cattle from different origins, dietary changes, social challenges, and environmental changes.(25, 26) Exposure time and compounding effects of different stressors including weaning result in immunosuppression affecting innate and adaptive immune responses and predisposing the calf to infectious bronchopneumonia.(27)

The effect of stress in weaning have been well characterized in calves, it is well known that abrupt weaning induces increased serum concentration of cortisol and norepinephrine,(24, 28) increase in peripheral catecholamines,(29) increase in heart rate,(30) increase in serum concentration of acute phase protein,(31) alteration in the ratio of neutrophils and lymphocytes,(24) and reduction on antioxidants enzymes activity of leukocytes.(32) All these factors induce oxidative stress and transitory reduction of immune functions between days 2 to 7

after weaning, that induce an impaired immune response of the affected animals and predispose to infections, including respiratory disease.(33)

Failure in the passive transference of immunoglobulins

Failure in the adequate absorption of immunoglobulins between the first hours of life results in the failure of passive transfer of immunoglobulins (FPTI). FTPI was determined to increase the risk of mortality in newborn calves, due to different condition including BRDC.(34) Different studies demonstrate that low IgG levels were associated with high morbidity or mortality prior to weaning,(35) and twice the odds for pneumonia.(36) Calves with high specific immunoglobulins were considered to be protected against respiratory diseases on a dairy operation.(37, 38) Additionally, the antibody status of calves was associated with the antibodies titer of dam, and the immune status with the presentation of respiratory disease.(39) Despite previous information, there is also research that did not demonstrate an association between antibodies titers and respiratory disease.(40, 41)

Time and distance of transportation

Transportation is one of the most recognized predisposing factors for BRDC; however, results from studies evaluating the effect of transport on BRDC presentation are inconsistent.(42-46) Based on the structure of the beef production industry in the U.S., a population of beef calves will be transported at least once in their lifetime to the next stage of production. For this reason, the identification and understanding of factors that predispose to BRDC during transportation are key to develop effective preventive measures. Transportation is an important stressor for cattle and depending on time and space available can result in increased BRDC morbidity by reducing

immune responses and increasing close contact between transported animals.(42) Greater transportation distance and time are positively correlated with BRDC morbidity in beef calves.(46) One study demonstrated a positive association between transport distance and BRDC morbidity; however, in that study other stressors (i.e., weaning, commingling, and environment) were not included in the analysis and may have confounded the results.(44) Results from another study did not find any difference in clinical presentation of BRDC in cattle transported over long distances versus cattle transported for short distances to the feedyard.(45) Results from a more recent study that evaluated the association between distances traveled from sale barns to commercial feedlots in the U.S. during 1997 and 2009, demonstrated a significant association between distance traveled and feedlot-calf morbidity and mortality for cattle that were transported ≥ 698 km.(43)

The effect of time of transportation on BRDC morbidity is not clear in the literature. One study compared total cattle transport time of 0, 12 and 24 hours on BRDC morbidity after feedyard arrival. Results from that study demonstrated a significant effect of short time transportation (12 hours) on BRDC morbidity and mortality compared with no transport (0h) and cattle transported 24 hours transportation.(47) Results from another study demonstrated a similar effect of short-term transport on BRDC morbidity and mortality.(48) Other factors such as the method of transportation (i.e., truck vs. train) and ventilation during transport (open with ventilation or closed) could play a role on the impact of transport on BRDC; however, results from different studies have not been able to demonstrate a significant effect of these factors on BRDC-associated morbidity and mortality after feedlot arrival.(20, 49, 50)

Commingling

Weaned calves entering the different marketing channels of the beef industry will commingle at some point with cattle from multiple origins. Commingling is an important risk factor for BRDC-associated morbidity and mortality because at this time the compounded stress/immunosuppression from weaning, transportation, and social challenge in the calf meets the greater risk of exposure to BRDC (usually viral) pathogens.(51) Results from one study demonstrated that receiving pens containing weaned beef steers from multiple sources following feedlot arrival demonstrated an increased BRDC incidence compared to receiving pens with single-sourced steers.(52) Another study, evaluated the effect of commingling heifers from multiple sources with single-sourced heifers on performance and BRDC incidence following feedlot arrival. Results from that study demonstrated an increased risk of BRDC re-occurrence after the second BRDC treatment in the group of multi-sourced heifers compared with the single-sourced group.(53) Commingling of recently weaned calves with cattle of unknown health status and from different sources is almost inevitable in the beef industry of the southeastern U.S. and exposure to respiratory viruses during this time can result in BRDC-associated morbidity and mortality losses to producers.

Environment

The majority of cases of BRDC are reported in the fall season; however, there is not an explanation for this association. It has been speculated that this maybe related with marketing time and increased movement of weaned and backgrounded beef calves into feedlots resulting in greater rates of commingling and greater pathogen pressure.(19, 54) Severe weather has been suggested as contributing factor for BRDC; however, results from some studies failed to

demonstrate an association between BRDC and severe weather;(54) or BRDC and daily meteorological changes.(55) Variation in temperature has demonstrated contradictory findings. While one study detected an association between temperature increases and BRDC;(56) results from another study demonstrated that increased temperature was associated with a decrease BRDC.(57) Other environmental variables including relative humidity, wind, and precipitation do not have an apparent significant effect on BRDC incidence;(56-58) however, dust and its size (exposure of cattle to particles between 2.0 to 3.3 mm in diameter) was determined to have a positive correlation with the BRDC presentation in one study.(59)

Source of animals

There are two important sources of cattle for beef production, cattle purchased directly from the farm of origin (single sourced) or those coming from auction barns and stocker/backgrounder farms (multiple sourced). Although newly arrived cattle have the greatest risk for BRDC regardless of source,(46, 56, 57) results from different studies have demonstrated a greater incidence of BRDC in cattle marketed or purchased from auction barns.(46) The greater incidence of BRDC in these cattle can be related with increased stress and greater pathogen exposure during multiple transportation, marketing, and commingling events.(52, 60, 61) As previously mentioned, commingling is one of the most important risk factors for BRDC morbidity and mortality and its impact is exponentially increased when multiple-sourced animals are placed together.(46, 50, 57, 62)

Age, weight, and gender

There is no clear association between age and BRDC. Results from one study suggested that younger cattle were more likely to be diagnosed with BRDC compared with older cattle.(63) In contrast, results from other studies demonstrated an important role of weight on BRDC presentation indicating that lighter-weight cattle have a greater risk of pneumonia following feedlot arrival.(46, 61, 64, 65) However, this is not entirely consistent in the literature as results from other studies suggest that cattle weight is not correlated with the presentation of BRDC or increase in BRDC treatment following feedlot arrival.(66, 67) The association between gender and BRDC presentation is inconsistent in the literature. Some studies have demonstrated an association between being a male and a higher risk of BRD;(35, 68) however, other studies have shown a positive correlation between being a heifer and the presentation of pneumonia.(19, 46)

Castration and dehorning

There is robust evidence of the negative effects that delayed castration has on beef production. Castration of male calves during the weaning and marketing stages decreases average daily gain (ADG) and increases acute phase proteins and cortisol production potentially increasing the risk for BRDC;(44, 69, 70) however, the correlation between castration and/or other veterinary procedures such as dehorning and BRDC presentation is not consistent among different studies. While a positive correlation has been demonstrated in some studies,(71) other studies have not demonstrated any effect of castration and dehorning on the presentation of BRDC.(72, 73)

Genetics

Genetic variation can confer cattle characteristics associated with resistance or susceptibility to different disease conditions including BRDC. Results from some studies suggest that the Braunvieh breed of cattle is more susceptible to BRDC compared with other breeds.(68, 74) The Angus breed is likely overrepresented in cattle from feedlot operations in the U.S. and therefore results from studies that identify it as a breed with greater or lower BRDC risk should be interpreted with care.(75, 76) Results from one study suggested that *Bos indicus* cattle are more resistant to BRDC compared with *Bos taurus* cattle.(58)

Microbiome

Recently the study of the gastrointestinal and respiratory microbiome and their changes/variation between healthy and sick cattle affected with different disease conditions have been studied.(77-79) The identification of beneficial bacteria such as *Lactobacillus* spp. in the respiratory tract of healthy cattle was associated with a healthy respiratory tract in one study;(79) however, the identification of bacteria such as *Mycoplasma bovis*, *Mannheimia haemolytica*, or *Pasteurella multocida* in the respiratory tract were associated with BRDC. (78, 79) One study of respiratory microbiome in calves demonstrated that the nasopharyngeal microbiota is highly influenced by the vaginal population of commensal bacteria from the dam, sharing more than 70% of bacteria including *Mannheimia* spp., *Moraxella* spp., *Bacteroides* spp., *Streptococcus* spp., and *Pseudomonas* spp.(77). It is possible that alterations in the normal commensal bacterial populations from the respiratory tract of cattle play a role on the clinical presentation of BRDC. The postulated mechanism of why commensal bacterial populations could protect against clinical disease include, antimicrobial action against other pathogenic bacteria, enhancing the epithelial barrier, and modulation of immune responses. Results from one study demonstrated the efficacy

of intranasal inoculation of *Lactobacillus* spp. in calves to prevent *Mannheimia haemolytica* colonization of the respiratory tract.(80)

Infectious agents

For many decades it has been known that different pathogens are involved in the presentation of BRDC. These agents include different types of viruses and bacteria. The most important bacteria associated with BRDC are gram-negative; however, *Mycoplasma* spp. and specifically, *Mycoplasma bovis*, are commonly involved in the presentation of BRDC. Infectious agents of the BRDC use different virulent and pathophysiological mechanisms to cause respiratory tract damage and disease and additionally can interact amongst them and act synergistically to lead to fatal outcomes.(3) In the next few paragraphs a small review of the most important infectious agents participating in BRDC morbidity and mortality will be performed.

Viral Pathogens

Bovine herpesvirus 1 (BHV-1)

Bovine herpesvirus 1 is a DNA alpha herpesvirus from the *Herpesviridae* family and *Alphaherpesvirinae* subfamily. There are three different subtypes of BHV-1, BHV-1.1, BHV-1.2a, and BHV-1.2b.(81) Subtype 1 has been associated with the disease known as infectious bovine rhinotracheitis (IBR) and it is usually associated with respiratory disease and reproductive losses (abortion).(82, 83) Bovine herpesvirus 1 is usually found in upper respiratory tract tissue

samples (nasal passages, nasopharynx, and trachea) and aborted fetuses and it is prevalent in Europe, North America, and South America.(83, 84) The subtype 1.2a is related with IBR, infectious pustular vulvovaginitis (IPV), infectious pustular balanopostitis (IPB), and abortions, and it is more prevalent in Europe and Brazil.(83, 84) The subtype 1.2b is associated with respiratory disease as well as with IPV/IPB, and it is prevalent in Europe and Australia.(85-87) Bovine herpesvirus 1 is most commonly associated with BRDC in U.S. cattle. The incubation period of BHV-1 following acute infection goes from 2 to 6 days, and clinical signs of affected animals include fever, anorexia, coughing, salivation, nasal discharge, conjunctivitis, inflamed nares and erosion of the muzzle area (red nose), inflammation and erosion of the upper respiratory tract (rhinitis, pharyngitis, laryngitis, tracheitis), and dyspnea in severely affected animals. Recovery usually takes 4 to 5 days if a secondary bacterial infection does not occur.(88)

One of the most important epidemiological aspects of BHV-1 is its ability to stablish lifelong latency after an animal has recovered from acute infection. After acute infection and replication in the upper respiratory tract, the virus travels intracellularly reaching the nervous system by the cells of the trigeminal ganglia. Once in the trigeminal ganglia cells' nucleus the virus silences all replication genes going into latency.(89) Several factors associated with stress such as weaning, transport, calving, severe weather and/or corticosteroid treatment can induce productive gene reactivation of latent BHV-1 leading to viral replication, reverse migration from the trigeminal ganglia via nerve cells and viral shedding.(90) The reactivation from latency follows three different events: 1) productive viral gene expression is detected in sensory neurons, 2) latency-related gene expression decreases, and 3) infectious virus is secreted from ocular and nasal cavities.(91-93)

Bovine Viral Diarrhea Virus (BVDV)

Bovine viral diarrhea virus are a group of enveloped single-stranded RNA pestiviruses from the family *Flaviviridae*. These viruses have a worldwide distribution, and infection in cattle can result in multiple clinical syndromes depending on the immunocompetence to the virus and stage of gestation. Although cattle are the natural host of BVDV, other domestic and wild species can be affected including but not limited to, sheep, goats, pigs, bison, alpacas, llamas, and white-tailed deer. Two genotypes, BVDV-1 and BVDV-2 and two biotypes, cytopathic (CP) and non-cytopathic (NCP) have been described.(94) Only NCP BVDV strains can generate persistently infected (PI) animals. The NCP biotype is most prevalent in the field and the most important source for viral infections.(95) Based on RNA sequencing of the first nonstructural protein region of the genome, there are 21 subgenotypes for BVDV-1 (a through u) and 4 subgenotypes for BVDV-2 (a through d). The most prevalent strains of BVDV in U.S cattle include BVDV-1b, BVDV-1a and BVDV-2a; however, during the last 20 years the most commonly BVDV strain isolated from PI cattle samples submitted to U.S. veterinary diagnostic laboratories is BVDV-1b.(96-98)

The clinical outcome of BVDV infections in naïve cattle depends on host factors such as immune status, pregnancy status, and gestational age of the fetus, and concurrent infections with other pathogens. Viral factors such as biotype, genotype, and antigenic diversity with respect to vaccine strains could play a role on clinical presentation. The different clinical syndromes described for BVDV infections in cattle include:

1. Acute or transient infection

In 70% to 90% of acute BVDV infections clinical signs are mild or absent (subclinical) in affected cattle. Acute (transient) BVDV infections usually occur in young cattle lacking specific immunity against BVDV. If present, clinical signs include mild diarrhea, depression, oculonasal discharge, anorexia, decreased milk production, and pyrexia. Direct contact with persistently infected cattle or, less frequently, with acutely infected cattle are the most common sources of infection.(99) Because BVDV is a lymphotropic virus and can induce apoptosis in white blood cells and especially in lymphocytes, BVDV infections in cattle regardless of strain, result in in leukopenia and lymphocyte depletion. This is one of the many mechanisms by which BVDV infections contribute to immunosuppression and predispose affected animals to secondary infections, especially with associated pathogens to BRDC.(100)

2. Severe Acute BVDV infection

The severe acute syndrome is characterized by a high morbidity and mortality rate. Mortality rate has been calculated as high as 25%, which varies between herds.(101) Clinical manifestations include diarrhea, fever, decreased milk production, and oral ulceration, with oral ulceration being more common in adult cattle.(99) Due to the oral lesions, the disease must be differentiated from mucosal disease, malignant catarrhal fever, vesicular stomatitis, papular stomatitis, foot and mouth disease, and bluetongue.(99) Viral isolation from outbreaks related with this specific clinical presentation were associated with NCP BVDV-2 strains;(102) despite these findings, not all BVDV-2

strains cause severe disease, and it is likely that some BVDV-1 strains are able to induce severe clinical disease under specific conditions.(99)

3. Hemorrhagic syndrome

The hemorrhagic syndrome is another acute presentation of BVDV. It was first described in both calves and adult cattle, with a severe depletion of platelet counts and high mortality.(103, 104) The clinical presentation is characterized by bloody diarrhea, epistaxis, petechial and ecchymotic hemorrhages, hyphema, bleeding from injection sites or insect bites, and affected animals also can present with fever, diarrhea, rumen stasis, and dehydration.(105) Experimental infection with NCP BVDV2 strains can induce hemorrhagic syndrome;(103, 106) there is also a report of colostrum deprived calves, that developed hemorrhagic syndrome with a BVDV-1b strain.(107) In addition to the low platelet count, leukopenia with a severe neutropenia is characteristic of affected animals; the infection of megakaryocytes at bone marrow level is believed to be the source of thrombocytopenia.(100, 108)

4. Reproductive syndrome

Bulls infected with BVDV can shed the virus through the semen during acute infection, but also some bulls can develop persistent testicular tissue infection and shed virus for a long period of time and up to 2.75 years without being a PI.(109) Acute BVDV infection in non-pregnant heifers and cows can result in infertility due to ovarian

inflammation or oophoritis.(110) Acute infection of pregnant cattle with BVDV can result in several conditions associated with reproductive loss including early embryonic death, abortion, calves born with congenital abnormalities, stillbirths, and most importantly calves, born persistently infected with the virus.(111) When BVDV infection of pregnant cattle occurs later than 150 days of gestation, potential outcomes include abortion, stillbirth, or calves born apparently normal but seropositive to the virus.(111-113) Results from previous research suggested that dairy calves born seropositive to BVDV (congenitally-infected) have a greater risk of developing severe illness compared with other calves.(113)

5. Persistent infection and mucosal disease

When infection of pregnant cattle with a NCP BVDV occurs between 45 and 125 days of gestation, the fetus can develop immunotolerance to the virus or persistent infection (PI). The process of immunotolerance is specific to the NCP BVDV strain causing the initial infection of the dam and involves negative selection of T and B naïve lymphocytes during the developing of the fetal immune system and function.(111, 114) Persistently infected calves are only immunotolerant to the specific NCP BVDV causing PI but can develop complete immune responses to infection with heterologous BVDV strains. From the epidemiological standpoint, PI animals are the most important source of BVDV for naïve cattle and cattle herds because they shed BVDV continuously in several body secretions including nasal and ocular discharges, urine, colostrum/milk, saliva, and feces.(115) In general, about 50% of PI calves die before 1 year of age; however, the

other 50% can make it to additional productive stages and adulthood.(115) Persistently infected calves that acquire an infection with a CP BVDV of great genetic similarity to the NCP strain causing PI develop a fatal disease known as mucosal disease. Persistently infected cattle affected with mucosal disease present with severe ulceration of the entire gastro-intestinal tract, hemorrhagic diarrhea, ulceration of the skin from the interdigital spaces, and death. Chronic mucosal disease, chronic mucosal disease with recovery, and delayed onset mucosal disease have also been described.(116)

Bovine Coronavirus (BCoVs)

Bovine coronavirus (BCoV) it is a single-stranded, positive-sense RNA beta-coronavirus from the family *Coronaviridae* that has tropism for the respiratory and gastrointestinal tract of cattle.(117) Infection of cattle with BCoV has been associated with three well-defined clinical syndromes including neonatal calf diarrhea (CD) in young calves, winter dysentery (WD)/hemorrhagic enteritis in adult cattle, and BRDC in cattle of various ages and stages of production.(118) Two to three subtypes have been identified and are associated with clinical disease in cattle.(119, 120)

The prevalence of BCoV has increased in the past years and infection with the virus has been associated with an increased risk of BRDC treatment weaned beef calves at feedlot entry.(121) In one study, 64% of dairy calves with diarrhea tested positive to BCoV in feces and in another study, 62% of calves with BRDC tested positive to BCoV in nasal secretions.(122, 123) Results from previous studies suggest that positivity for BCoV in fecal or nasal secretion samples significantly increases the risk of diarrhea and BRDC in dairy and beef calves.(123-125)

The presence of the virus in nasal secretions of weaned beef calves on the day of arrival at the feedlot increased the likelihood of developing clinical BRDC 45 times during the first 3 weeks of feeding.(125-127) Additionally, BCoV has been recovered from lungs of cattle affected with *Pasteurella* spp. infection and BRDC.(128, 129) Results from different studies demonstrated that cattle arriving with high serum antibody titers against BCoV at the feedlot were less likely to shed BCoV, seroconvert, or develop clinical BRDC.(128, 130, 131)

Bovine Parainfluenza-3 virus (PI3)

Parainfluenza 3 virus is a respirovirus of the subfamily *Paramyxovirinae*, order Mononegavirales, family *Paramyxoviridae*. This is a single-stranded, non-segmented, negative-sense RNA virus involved in BRDC.(132) The virus replicates in the upper respiratory tract and usually acts in synergy with other pathogens in the presentation of clinical BRDC.(132) Results from studies in which experimental infection of cattle with the virus was performed have demonstrated variable results. While in some studies, challenged cattle develop pneumonia, in other studies challenged cattle remained asymptomatic.(133) The virus replicates in the upper respiratory tract (trachea) and causes damage of the mucocilliary systems compromising clearance of debris and other respiratory pathogens from the lower respiratory tract.(134-136) Common clinical signs following experimental infection with the virus include fever from day 2 to 10 post-infection, cough, nasal discharges, as a consequence of rhinitis, and anorexia.(133, 137) Affected cattle usually recover approximately 10 days following presentation of clinical signs.(132, 138) The study of pathological lesions associated with PI3 infection in cattle is challenging because the virus is usually associated with other respiratory pathogens in natural

occurrence of BRDC. Additionally, experimental challenge studies in cattle have produced variable clinical and pathological results.(133, 139)

Bovine Respiratory Syncytial Virus (BRSV)

Bovine respiratory syncytial virus is considered one of the most important infectious agents associated with the presentation of BRDC. The seroprevalence of the virus in U.S. cattle is high, and BRSV infection has been associated with variable levels of BRDC morbidity and mortality in beef and dairy calves. The virus is a pneumovirus closely related to the human respiratory syncytial virus (HRSV). BRSV is a negative sense, single stranded RNA virus. Based on differences in the structure of surface proteins, four different serogroups of BRSV have been previously described. A thorough review on the biology and pathophysiology of BRSV can be found in the following chapter of this dissertation.

Bacterial pathogens

Mannheimia haemolytica

M. haemolytica is a gram-negative aerobic coccobacillus from the family *Pasteurellaceae* and it is the most commonly isolated bacteria from BRDC-affected cattle.(16) There are at least 12 capsular serotypes with serotype A1 and A6 most commonly isolated from lungs affected with *M. haemolytica* infection.(140, 141) *Mannheimia haemolytica* is a commensal microorganism associated with the upper respiratory tract of cattle and isolation of this agent from upper respiratory tract samples from healthy and BRDC-affected cattle is not uncommon;

however, its isolation rate increases under stressful conditions (i.e. weaning), transport, and following viral infections .(140, 141) Replication of respiratory viruses such as BHV-1, PI3, BCoV, and BRSV in the upper respiratory tract (i.e. trachea) leads to immunosuppression and damages the mucocilliary apparatus affecting clearance and allowing virulent forms (A1, A6, etc.) of *M. haemolytica* to reach the lungs and cause severe pneumonia.(142, 143)

M. haemolytica has different virulence factors associated with lung pathology. The capsular polysaccharides affect the phagocytic ability of neutrophils and compromise the opsonizing effect of antibodies.(143, 144) The lipopolysaccharide (LPS), specifically the lipid A portion activates the acute inflammatory response syndrome including macrophage activation, neutrophil migration, inflammatory cytokine (IL-1β, IL-6, IL-8, TNFα) release, endothelial changes and inflammatory mediators (prostaglandins, leukotrienes, thromboxanes). This results in fever and signs of shock including decreased cardiac output, hypotension, reduced tissue perfusion, peripheral vascular edema, multiorgan failure and eventually death.(143) The most important virulence factor of *M. haemolytica* is a cytotoxin known as leukotoxin. Leukotoxin lyses neutrophils in affected tissues and promotes the release of proteolytic enzymes and radical species of oxygen (i.e., peroxide, etc.) that subsequently cause massive tissue damage and necrosis of the lung. This results in coagulation necrosis and fibrin deposition.(140, 143)

Pasteurella multocida

P. multocida is a gram-negative aerobic coccobacillus from the family *Pasteurellacea*. Five capsular serogroups and 16 serotypes have been described for *P. multocida* with A:3 and D:3 being most isolated from cattle and especially from young calves.(145) Similar to *M.*

haemolytica, *P. multocida* is part of the commensal microbiome of the nasopharynx of healthy cattle; however, the rate of isolation of *P. multocida* from upper respiratory tract samples of calves affected with pneumonia can be as twice as high when compared with healthy calves.(146, 147) Affected animals are usually young calves undergoing stressful events such as weaning and/or transport. Clinical signs of calves with *P. multocida* infection include depression, decreased appetite, abnormal lung sounds, tachypnea/dyspnea, and fever.(148)

Pasteurella multocida infections are usually comorbidities with other respiratory viruses and/or bacteria such as *Histophilus somni* and *Mycoplasma bovis*, but it is rarely isolated alone from lung tissues of affected animals. However, a significant increase in the number of fatal cases of pneumonia in cattle from feedlots where *P. multocida* was isolated from affected lungs have been recently reported.(140, 149, 150) The increase in the number of *P. multocida* BRDC cases may be related with a shift in the age at which beef calves arrive to the feedlot. The increase in the practice of backgrounding at the farm of origin, marketing single-sourced beef calves and greater size of weaned beef calves in the U.S. increases the changes of younger animals arriving to feedlots could explain the reason why *P. multocida* has become more frequently isolated in cases of BRDC.(151, 152)

Histophilus somni

Histophilus somni is a commensal, gram-negative, non-spore forming bacteria of the family *Pasteurellaceae* that can be found in the nasopharynx of healthy cattle.(16) Due to its ability to reach circulation and cause vasculitis and vascular thrombi, infection of cattle with *H. somni* has been associated not only with BRDC but with other diseases such as septicemia,

meningoencephalitis, endometritis, abortion, pneumonia, pleuritis, laryngitis, otitis, conjunctivitis, myocarditis, mastitis, and polyarthritis.(153) *Histophilus somni* is not commonly isolated from nasal secretion samples of healthy, single sourced calves that have not been commingled with other cattle; however, it can be found following stressful events (i.e., transport) and arrival to the feedlot.(154) High levels of serum neutralizing antibodies against *H. somni* may reduce the possibility of isolation.

Several virulence factors such as the outer membrane lipoproteins (OMPs) and LPS, as well as the ability to form biofilm, contribute to the pathophysiology of *H. somni* infections in cattle. The OMPs play an important role in the induction of vasculitis and vascular thrombi in addition to driving T-helper 2 (TH2) responses with high production of specific IgE and histamine in the host. These responses could increase the severity of clinical disease and worsen previous viral infections specifically with BRSV. Results from previous studies suggest that dual BRSV and *H. somni* infections of cattle leads to more severe clinical disease and lung pathology compared with individual infections with either pathogen.(155) Additionally, results from a recent study demonstrated that high-risk feedlot calves vaccinated with an IN MLV BRSV vaccine at arrival had an increased carriage of *H. somni* in the nare compared with MLV BRSV parenterally vaccinated calves. (156) The mechanism by which previous BRSV infections increase *H. somni* pathogenesis in calves is believed to be associated with the ability of BRSV to induce greater *H. somni*-IgE and histamine production during dual infections.(155, 157) Similar to cases of *M. haemolytica* infections, the presence of LPS and induction of endotoxemia are important pathogenic factors of *H. somni* .(158) Respiratory disease caused by *H. somni* is considered the window of entrance and opportunity to other forms of clinical presentation of the

disease; however, *H. somni* is usually is isolated from the lungs of affected cattle in combination with *P. multocida*, and *M. haemolytica.*(158)

Mycoplasma bovis

Mycoplasma bovis is a member of the genus *Mycoplasma* and the class Mollicutes. This is a facultative anaerobe without a cell wall but a trilayered membrane instead.(16) The role of *M. bovis* in the pathophysiology of the BRDC is not completely clear;(159) however, it has been more frequently isolated from lungs of cattle affected with BRDC compared with healthy cattle.(79) Additionally, experimental infection of calves with *M. bovis* resulted in pneumonia in one study.(160) *Mycoplasma bovis* is mostly recognized as a secondary pathogen within the BRDC complex, and clinical signs of chronic pneumonia in calves are usually observed following primary infection of *M. haemolytica*, *P. multocida* and *H. somni*. Respiratory signs of calves affected with *M. bovis* pneumonia can include fever, tachypnea, inappetence, head tilt, and sometimes respiratory distress. Other important clinical signs may include signs of otitis media (head tilt) in young animals or as arthritis or tenosynovitis in newly arrived feedlot animals.(161, 162)

The incidence of the disease in some herds varies from 0% to more than 90%.(161, 163) Colonization of the respiratory tract of calves by *M. bovis* in endemic farms occurs early in life and transmission occurs by aerosol (close contact) and/or contaminated milk or colostrum.(159) In most cases early colonization of the respiratory tract of young calves with *M. bovis* does not result in clinical disease but infected cattle can remain as carriers or develop clinical disease later in life following stressful events (i.e., weaning, transport, primary viral or bacterial infections).

After entering the respiratory tract, *M. bovis* moves through infected cells and can access the blood stream. Once in circulation, *M. bovis* can localize in different tissues/organs causing different clinical syndromes (i.e., arthritis, otitis media, mastitis, etc.). Once an animal is infected with *M. bovis*, it can become a carrier and shedding for long periods is expected as the infection persist in macrophages and *M. bovis* is effectively evading immune responses.(162) *Mycoplasma bovis* has 5 different variable surface lipoproteins that facilitate its ability to evade host immunity and cause persistent infection.(161)

Bibersteinia trehalosi

Bibersteinia trehalosi is a gram-negative coccobacillus of the family *Pasteurellaceae*. It causes pneumonia, systemic disease, and multiorgan failure in sheep. These bacteria are considered a rare cause of pneumonia in cattle; however, peracute and fatal pneumonia has been reportedly associated with multi-drug resistant (MDR) *B. trehalosi* infection in recently weaned beef calves.(164) Attempts to reproduce severe clinical disease following an experimental challenge model in calves were not successful and the role of this agent in the BRDC is not completely understood.(164)

Economic impact of BRDC

The economic impact of BRDC in the U.S. feedlot cattle industry has been estimated to be in the order of $23.60 per clinical case and close to $55 million annually.(13) The associated costs include culling and death of animals, reduced of performance, veterinary fees, antibiotic treatment, labor and other management costs.(1) The negative impacts of BRDC on performance

are far prolonged beyond response to treatment and abatement of clinical signs increasing the economic impact of the disease.(2)

Pre-weaning BRDC

During pre-weaning period, a phase of production that in U.S. beef cow-calf production systems typically occurs from birth to 6-8 months of age, previous reports indicate a 20% of prevalence of BRDC in calves and mortality rates between 3.3 and 23.6%;(151, 165) however, the incidence of the disease can vary between years, among different regions, and among different farms. The peak incidence was reported between 80 to 120 days of age and usually occurs during the summer.(151)

The main source of profit and income for U.S. cow-calf operations comes from the number of weaned calves sold and the body weight (live weight) of these animals. Disease or reduced health negatively impact weight gain and could result in an overall reduction of the total number of calves sold due to mortality. The costs associated with treatment and labor contribute to the economic impact of pre-weaning calf pneumonia on cow-calf operations with a high incidence of the disease.(2) Respiratory disease has been reported as an important cause of death in beef calves 3 weeks of age or older.(166, 167) Additionally, BRDC has been associated with 16.4% of the total mortality during the pre-weaned period in U.S. beef calves.(12, 168) The impact of BRDC on growth and performance of pre-weaned beef calves has not been clearly studied or defined.(2, 14)

In dairy calves, BRDC is the leading cause of morbidity and mortality during the pre-weaning period.(169, 170) In one report, the BRDC prevalence in pre-weaned dairy calves from U.S. dairies was around 23% with a mortality rate of 19%.(171) Results from some studies suggest that despite successful treatment of calves for BRDC, negative long-term productive impacts are observed when compared with healthy untreated herd-mates.(172, 173) One study demonstrated an average short term cost of $42.15 per dairy calf affected with BRDC. The cost included the use of anti-inflammatory drugs, antibiotics and other treatments. Additionally, the same study demonstrated an increase in 3% of milk feeding for affected animals and a 10 to 15% increase in production cost to the use preventive BRDC measures.(174)

Post-weaning BRDC

The bovine respiratory disease complex is recognized as the most important cause of morbidity and mortality in weaned beef calves.(12, 167, 175) According to official USDA statistics, BRDC has a prevalence of 16.2% and is the most important health problem in U.S. beef cattle. Several factors play a role in the presentation of BRDC post-weaning in beef calves including weaning, transport, and commingling. The majority of BRDC cases in weaned beef calves are observed during the first 60 days after stocker of feedlot arrival.(168) The cost of an individual case of BRDC in feedlot calves that survive the feeding phase can be from $45 to $92 per head, and 50–60% of this cost is associated with reduced performance.(176-178)

Treatment impact

It was estimated that a single BRDC treatment with antibiotics in the feedlot cost $23.60 per animal according with the USDA in 2011. This value does not include the cost of labor or reduced performance. Based on U.S. cattle inventory, animal turnover twice a year and a prevalence of 16%, the total annual BRDC treatment cost in the feedlot industry can be calculated around $88.7 million;(168) however, this does not account for cattle that require more than one treatment. More than one BRDC treatment in feedlot cattle can result in $70 to $90 additional individual net return losses (due to treatment cost) on top of other costs associated with decreased performance, additional days on feed, decreased hot carcass weight, ribeye area and quality grade.(179, 180) On the other hand, the use of metaphylactic antibiotic treatment significantly reduces the clinical presentation of BRDC following feedlot arrival; however, the cost of this practice is much more than the $532 million for treatments per se, it also includes the greater risk of promoting the developing multidrug resistant BRDC bacteria.(181, 182)

Performance impact

Calves affected by BRDC experience a reduction of 0.07 kg on average daily gain (ADG) and a 3 to 7% reduction in performance compared with healthy calves.(67, 183, 184) Additionally, results from different studies have demonstrated that calves affected with BRDC have a reduction in their weight at slaughter of 4 to 11 kg, reduction of 3 to 8.6 kg on hot carcass weight, and a reduction in the intramuscular fat compared with non-affected calves.(177, 185)

Diagnostic impact

Clinical diagnosis of BRDC requires constant monitoring of the animals at risk, and this requires inherent expenses on training, labor, and facilities; however, an accurate and early diagnosis of BRDC allows for early treatment and improved outcomes. Currently, different clinical scores and calf-side diagnostic tests with different sensitivity and specificity values are available. Results from one study suggested that using tests with a greater specificity more than sensitivity can decrease the negative economic impact of BRDC by accurately identifying animals that do not require treatment or intervention.(183) Based on current literature, a combination of diagnostic tests with high Se and Sp values (i.e., clinical scores and thoracic ultrasound examination) have been suggested to improve the identification of calves that require BRDC treatment at feedlot arrival without sacrificing processing efficiency.(184, 186)

Bovine Respiratory Syncytial Virus

The bovine respiratory syncytial virus (BRSV) is a virus associated with the bovine respiratory disease complex. This virus is highly prevalent in dairy and beef cattle in North America and probably around the world.(187, 188) The virus was first associated with respiratory disease in cattle in the 1960s; at that time, antibodies for the human respiratory syncytial virus (HRSV) were found in bovine serum. During the 1970s, BRSV was first isolated and identified from a respiratory disease outbreak in European calves and by 1974, BRSV was first identified in the U.S. in the states of Iowa and Missouri, U.S..(189)

Epidemiology

Outbreaks of respiratory disease in calves associated with BRSV commonly occur during the fall and winter months in the northern hemisphere; however, cases have been reported during the summer months too.(189) Risk factors associated with the presentation of clinical disease include movement of cattle, overcrowding and close contact between animals, and fluctuations of ambient temperature.(189) In calves, BRSV infection and disease has been associated with failure in the transfer of passive immunity, rapid decay of maternal antibodies, introduction of new cattle into the herd, and shedding of BRSV by adult cattle with subclinical/chronic BRSV infections.(190-192) Like other viruses that affect the respiratory tract, BRSV is transmitted from infected to naïve cattle horizontally. Cattle are considered the primary host and adult cattle are thought to be the main reservoir of BRSV for younger calves by repeatedly developing subclinical infections and shedding the virus.(192, 193) Based on results from one study that demonstrated an 11% variation in the genome of different BRSV isolates from the same herd over time, re-introduction of the virus more than latency/persistency of chronic infections is believed to be the most likely mechanism used by BRSV to circulate among cattle herds.(194) One study following BRSV seropositive cattle over a period of 100 days in 6 different dairy herds did not demonstrate cross-transmission of BRSV between herds;(195) however, the potential of persistency of BRSV within herds was suggested. Results from other studies demonstrated the presence of BRSV in tracheobronchial and mediastinal lymph nodes of calves experimentally infected with the virus up to 71 days post-infection.(195, 196); however, attempts to reactivate viral replication and shedding following immunosuppressive treatment in previously BRSV infected cattle failed to produce positive results.(196)

Bovine respiratory syncytial virus is a common virus among cattle populations worldwide with morbidity rates reported to be as high as 80% and mortality rates ranging between 2-3% to up to 20%.(192) The seroprevalence of BRSV at the farm level in beef and dairy operations around the world varies between different countries; however, in North America, BRSV seroprevalences between 60-80% in the United States, 22-53% in Canada, and 52-91% in Mexico have been reported.(189, 197) In Europe, a seroprevalence between 40-100% has been reported in Sweden and a seroprevalence of 54% has been reported in Denmark.(198-200) Seroprevalence in cattle from South African feedlots have been reported to be approximately 43%, while in U.S. feedlots BRSV seroprevalence can be as high as 95%.(201, 202)

Pathogenesis

Virus biology

BRSV is an enveloped, positive sense, non-segmented, RNA virus from the genus Orthopneumovirus, within the family *Pneumoviridae*. The cytopathic effect of the virus within the lung parenchyma results in syncytial cell formation and that is where its name comes from. The genome of BRSV encodes eleven proteins.(203)

1. Transmembrane glycoproteins
    a. Attachment glycoprotein (G)
    b. Fusion protein (F)
    c. Small hydrophobic protein (SH)
2. The matrix protein (M)

3. Nucleocapsid
    a. The nucleoprotein (N)
    b. Phosphoprotein (P)
    c. RNA polymerase (L)
    d. M2-1
    e. M2-2
4. Two non-structural proteins
    a. NS1
    b. NS2

Replication and viral recognition

Replication of BRSV was demonstrated in bronchi, bronchiolar and in alveolar epithelial cells, within 3 to 5 days post-infection in addition to histopathologic changes including syncytial formation in bronchiolar walls and alveoli.(204) In infants, the human respiratory syncytial virus (HRSV) has been found in small bronchiole epithelium and type 1 and 2 alveolar pneumocytes post-infection.(205) A proinflammatory response including different chemokines and cytokines follows BRSV infections of the lungs in cattle. (203, 206) Different studies report an up-regulation of interleukins (IL); IL-12, IL-6, IL-18, IL-8, Interferons (IFN), IFNγ, IFNα and IFNβ and tumor necrosis factor-α (TNFα) in addition to activation of RANTES (regulated upon activation, normal T cell expressed and presumably secreted), Monocyte chemoattractant Protein-1 (MCP-1) and Macrophage inflammatory protein-1 alpha (MIP-1α).(203, 206) These

molecules activate the inflammatory cascade that leads to cell migration, alveolar and interstitial edema, and emphysema characteristic of severe BRSV infections in calves.

Experimental infection of laboratory animals with human respiratory syncytial virus (RSV) has demonstrated activation of different Toll-like receptors (TLR 2 and TLR6) on hematopoietic cells and promoting neutrophil migration and dendritic cell (DC) activation.(207, 208) In addition, in HRSV-infected mouse cells it was demonstrated that activation of the TLR3 pathway resulted in additional chemokine upregulation.(209) Similarly, activation of TLR3 and expression of MCP-1, MIP-1α and IL-10 mRNA in γ/δ T cells has been demonstrated in bovine cells following BRSV infection.(210)

Toll like receptor 4 (TLR4) and its co-receptor CD14 have been shown to interact with HRSV in both human and mouse immune cells. The F protein of HRSV activates the TLR4/CD14 complex resulting in NF-$\varkappa\beta$-mediated inflammation in both mouse and human cells.(211) Activation of TLR7 in HRSV-infected mouse macrophages results in increased expression of interleukin IL-12 and IL-23.(212) Similarly, bovine TLR7 activation has been demonstrated in vitro following BRSV infection of bovine immune cells; however, its role on the inflammatory cascade initiated by BRSV infection has not yet been determined.(213)

Following HRSV recognition, macrophages and epithelial cells release cytokines and chemokines such as TNFα, IL-1β, IL-6, IL-8 MIP-1α and RANTES. The main function of these is to initiate an inflammatory response, and depending on the magnitude of this initial inflammatory response the severity of BRSV infection and disease in the host (i.e., human

infants) varies.(214) Similarities in the activation of the inflammatory cascade following BRSV infection in cattle have been reported. Infection with BRSV in calves results in the release of RANTES, MIP1α–2α–3α, MCP-2 mRNA TNFα and IL-1 β in dendritic cells and monocytes. (215, 216) Some of the functions of important inflammatory mediators that play a significant role during BRSV infection and disease in cattle are described below.

- IL-1 β orchestrate the proinflammatory response.(215, 217-219)
- IL-1 β and IL-18 enhance IL-12 and IFNγ production and regulate innate and acquired immune responses.(220)
- Type I IFNs have antiviral effects on neighboring uninfected cells; however, BRSV can display a degree of resistance to the anti-viral effects of type I IFNs than other paramyxoviruses.(221, 222)

Modulation of innate immune responses

As previously mentioned, following BRSV infection there is a group of proinflammatory cytokines and chemokines secreted that are involved in the pathological events of the disease. Results from studies in humans have demonstrated that the NS1 and NS2 proteins of HRSV suppress type 1 IFN production and therefore reduce the ability of epithelial cells to prepare for imminent viral infections.(222) The suppression of type 1 IFN production facilitates viral adherence and fusion to mucosal cells. Additional research on the role of the G protein demonstrates that it can modulate innate and adaptive responses by interacting with the immune system; secreted G protein might act as a decoy by binding to neutralizing antibodies,(223) the

conserved cysteine region has homology with the CX3C chemokine receptor facilitating infection,(223) and by interaction with L-selectin, annexin II, and surfactant proteins.(215, 224-228) The suppression of innate immune responses by viral proteins facilitates entrance of the virus to the host cell and viral replication.

Antigen presentation

Antigen uptake and presentation is a function of antigen presenting cells (APC). In mammals, monocytes, macrophages, and dendritic cells are the most common APC. Dendritic cells trap antigens and migrate from peripheral tissues to local lymph nodes becoming afferent lymph dendritic cells (ALDC). Bovine ALDC can be subdivided into two main subpopulations, those expressing high levels of CD172a (SIRPα) and those expressing low levels or no CD172a. Although both ALDC subsets can take up and present BRSV antigens, the CD172a^{high} population is more efficient in presenting BRSV antigens to resting, naïve and memory CD4+ T cells compared with the CD172a$^{low/neg}$ population of ALDC.(229)

Modulation of adaptive immune responses

Infection with BRSV does not induce life-long immunity; however, reinfection of calves with BRSV is usually subclinical or results in mild clinical disease.(230) Based on available literature, the peak incidence of severe BRSV disease in calves occurs between 1 and 3 months of age when calves may still have some maternally derived immunity which can contribute at some degree with clinical protection.(231) Following natural BRSV infection or vaccination, B

cells produce neutralizing antibodies. Most of humoral immune responses including neutralizing antibodies are directed against BRSV surface glycoproteins F and G.(232) The F protein is highly conserved between strains and amino acids 255-275 and 417-438 of the F protein form the most common epitopes (232, 233). Neutralizing antibodies against the F protein prevent syncytia formation. On the other hand, the G protein of BRSV is less conserved between strains and it is unclear if neutralizing antibodies against the G protein play a significant role on protection against BRSV reinfection.(234)

Initiation of cell mediated immunity has been related with different viral proteins. Epitopes for $CD4^+$ T cells were identified on F and G protein; while for $CD8^+$ T cell the epitopes were found on M2, F, and N protein.(235, 236) After viral infection, $CD4^+$ and $CD8^+$ T cells increase in lung tissue and lymph draining the lungs of infected calves.(204, 237-239) Cytotoxic T cells can be detected in the lungs and peripheral blood, 7 to 10 days after infection;(240) IL- and IL-13 also can be found on days 3-4, and INF between days 5-8.(238) $CD8^+$ T cells were demonstrated to play an important role for recovery from infection, by contributing to elimination of the virus in the upper and lower respiratory tract;(224, 240, 241) which is associated with an increase in INF production.(237) On the other hand, $CD4^+$ T cells were associated with more extensive pulmonary pathology in calves; as in humans, in which depletion was associated with prolonged virus shedding and increased disease severity.(241-243)

Dynamics of antibody responses

After infection of naïve calves, BRSV-specific immunoglobulin M (IgM) and immunoglobulin A (IgA) can be detected in serum, bronchoalveolar lavage (BAL), and nasal secretions during the first 8 to 10 days.(244) BRSV-specific IgA remains detectable in nasal secretions for at least 3 months. Serum immunoglobulin G1 (IgG1) can be detected by 2 weeks after initial infection, whereas IgG2 antibodies are not detected until 1 to 3 months post-infection.(244, 245) BRSV-specific serum IgG persists for at least 3 months following initial infection.(244, 245) The induction of serum and mucosal antibodies is suppressed in colostrum-fed calves with high levels of maternally-derived BRSV antibodies; however, following reinfection with BRSV and even in the absence of initial systemic or local antibody responses, calves naturally exposed to or vaccinated against BRSV in the face of maternal antibodies (IFOMA) can develop an anamnestic systemic and mucosal IgA responses and demonstrate a reduction in the severity of clinical disease and viral shedding.(244, 245)

Effect of age on immune responses

Studies in calves suggest an age-related difference in TNF-α production that may play a role on the immune response against BRSV infection. Neonatal calves, without maternally-derived antibodies and experimentally infected with BRSV at 1 day of age, had fewer neutrophils in the lung and less severe clinical signs compared with calves infected at 6 weeks of age.(246) The observed changes were associated with more extensive virus replication and lung consolidation in addition to a lower level of TNF- α and BRSV-specific antibody responses in calves infected with BRSV at 6 weeks compared with calves infected with BRSV at 1 day of age.(246) Based on this information, it is possible that age-associated ability to produce TNF- α

plays an important role in the pathogenesis of BRSV infections in calves and could explain why less severe clinical disease is observed in calves less than 1 month of age.

Additionally, in ruminants the Y/δ TCR T cells are a major component of the circulating pool of lymphocytes. In contrast, circulating Y/δ T cells represent less than 5% in humans and mice.(247) Although the response of bovine Y/δ T cells to BRSV infection has not been investigated in calves, the Y/δ T cells of infants infected with HRSV produced less IFNγ and more IL-4 and it is possible these cells play an important role in the immunopathology of BRSV infections in calves led by Th2 responses. Healthy calves' Y/δ T cells exposed to BRSV either demonstrate increased levels of MCP-1 and MIP-1 α but not IL-10 or IFNγ, or increased expression of IL-10 and IFNγ.(210)

In a mouse HRSV infection model, the depletion of pulmonary Y/δ T cells resulted in reduced lung inflammation and an increased lung RSV titres, suggesting that Y/δ T cells have proinflammatory and antiviral effects.(248) In contrast, depletion of bovine Y/δ T cells did not affect virus clearance from the respiratory tract, or affect pulmonary pathology in calves infected with BRSV;(239, 241) however, increased levels of specific IgM and IgA in bronchoalveolar fluid (BALF) of infected calves was observed 10 day after infection.(241). Additionally, bovine Y/δ T cells spontaneously secrete IL-10 and can inhibit antigen-specific and nonspecific proliferation of CD4+ and CD8+ T cells in vitro potentially promoting a Th2 response to BRSV infection and contributing to the pathophysiology of the disease in young calves.(249)

Co-infections

Different co-infections with BRSV can exacerbate the pathogenesis of BRSV infection and disease in cattle. A co-infection of cattle with BRSV and BVDV resulted in depression of alveolar macrophage function increasing the risk of secondary bacterial infections based on results of one study.(250) Results from another study demonstrated that exposure of cattle to *Micropolyspora faeni* prior to experimental infection with BRSV was associated with greater BRSV IgE expression and increased pathogenesis and clinical disease.(251) Experimental infection of cattle with BRSV prior *Histophilus somni* resulted in more severe clinical disease, greater serum titers of H. somni IgE and IgG, and greater isolation of *Histophilus somni* from the lungs at necropsy when compared with experimental infection with only one of these pathogens.(155) Recently, results from one study demonstrated that vaccination of high risk beef calves with an IN MLV BRSV vaccine at feedlot arrival resulted in increased detection of *H. somni* in nasal secretion samples during the first 28 days of feeding compared with calves vaccinated with a parenteral MLV BRSV vaccine.(156)

Clinical disease

The clinical presentation of cattle infected with BRSV can vary from subclinical to fatal, depending on age, immunological status, predisposing factors and level of specific immunity.(7, 252, 253) Clinical and pathological signs of natural BRSV infections in cattle are commonly enhanced by the presence of other viral and bacterial agents of the BRDC. Following experimental infection of calves with BRSV, clinical signs are usually observed between days 4

to 6 post-infection; however, in naïve calves, clinical signs could be observed as early as 2 days post-infection. Common clinical signs of BRSV infection in cattle include fever, depression, decreased appetite, and increased respiratory rate. More severely affected animals can develop cough and nasal discharge.(254, 255) In some cases, BRSV infection can result in dyspnea, respiratory distress (open mouth breathing, abducted elbows), subcutaneous emphysema, and sudden death following a peracute infection; however, in the majority of BRSV infections, affected animals recover around 10 days.(256)

Diagnosis

Antibody detection

Serology and detection of BRSV antibodies in other body fluids are not diagnostic of current BRSV infection and disease; however, antibody detection can be used as a measure of previous BRSV infection or vaccination. Identification of BRSV-specific antibodies in serum can be performed by either enzyme-linked immunosorbent assay (ELISA) or serum neutralization (SN).(192). It was determined that serum ELISA was 95% specifici and 92% sensitive for the detection of BRSV-specific antibodies when compared to virus neutralization as a gold standard.(257) Utilization of ELISA for detection of BRSV-specific antibodies in nasal secretions can be performed for detection of IgG, IgG1, IgG2, and IgA.(258)

Reverse transcription - Polymerase chain reaction (RT-PCR)

Reverse transcription polymerase chain reaction (RT-PCR) is a sensitive and reliable technique for the detection of BRSV genetic material in respiratory tract samples of cattle. One study compared RT-PCR for the detection of BRSV in dairy calves with clinical respiratory disease using four different samples [nasal secretions (NS), deep nasopharyngeal swabs (NPS), transtracheal wash (TTW), and BALF].(259) Results from that study demonstrated that 9.4% of the calves were RT-PCR positive for BRSV in NS samples, 13.8% were positive in NSP, 17.4% were positive in TTW and 16% were positive in BALF.(259) In this study, the agreement among BRSV RT-PCR results from different samples varied as follows, TTW vs. BAL was very good at 92.9%, TTW vs. NS was moderate at 60.9%, and TTW vs. NPS was good at 78.6%.(259) Another study compared RT-PCR and immunofluorescence (IF) for the detection of BRSV on NPS samples and results indicated that 31/35 (89%) of isolates were RT-PCR positive compared with 23/35 (66%) in the IF group.(260) In the same study, the authors determined that nested RT-PCR for BRSV detection was 10 times more likely to determine positivity in individual samples compared with RT-PCR or virus isolation.(260)

Gross pathology

Gross postmortem evaluation following BRSV infection is characterized by collapsed cranioventral lobes of the lungs that may be dark red-purple due to atelectasis.(261) Similar areas of consolidation may be noted throughout the cranial, middle, or accessory lobes.(261) The caudodorsal portion of the lungs may fail to collapse, and if emphysema is present, the lungs may appear pale.(262) The nasal meatus, trachea, bronchi, and bronchioles may contain foamy or mucopurulent discharge.(263) A demarcation between emphysematous and consolidated lung

parenchyma is often apparent.(263) If bullae or emphysematous lesions have ruptured, pneumothorax, pneumomediastinum, or pneumopericardium may be present.(245) Along with these lesions, mediastinal lymph nodes are often enlarged.(245)

Histopathology

Microscopic examination reveals bronchointerstitial pneumonia, necrotizing, bronchiolitis, type II pneumocyte hyperplasia, syncytia formation, and exudative alveolitis.(262) Necrotizing bronchiolitis is characterized by necrotic ciliated and non-ciliated respiratory epithelium.(245) Fibrin casts, alveolar macrophages, seroproteinaceous fluid, and occasional neutrophils may be noted within the alveoli.(262, 263) Syncytial cells project from the bronchiolar wall and occasionally in the alveoli.(245) Eosinophilic intracytoplasmic inclusion bodies may be noted in syncytial cells.(191) Evaluation of tracheobronchial lymph nodes reveals prominent follicles within enlarged cortices along with expanded parafollicular areas likely due to lymphocytic hyperplasia.(245)

Tissue identification

Detection of BSRV in lung tissue samples can be performed using RT-PCR, immunohistochemistry (IHC), immunofluorescent antibody (IFA) staining, and direct fluorescent antibody techniques (d-FAT) on fresh, frozen, and formalin-fixed tissues.(245, 262) Based on results from one study, there is a 100% correlation between RT-PCR and d-FAT results in lung tissue samples from cattle positive to BRSV.(264)

Treatment and prevention

Treatment

Treatment for BRSV infection consists of supportive care, reducing the inflammatory response and preventing secondary bacterial infections. The use of anti-inflammatory therapy including non-steroidal anti-inflammatory drugs (NSAIDs) or steroids is common in cases of severe disease characterized by dyspnea or respiratory distress. Antimicrobials to treat or prevent secondary bacterial infection may be indicated, although these are not invariably required. Depending on the severity of the clinical disease, some patients will need insufflation with intranasal oxygen, and diuretic therapy for reduction of pulmonary edema in an aim to reduce respiratory distress. Overall, the prognosis for animals with uncomplicated BRSV infection is good; however, in severe peracute cases presenting with signs of respiratory distress the prognosis is guarded to poor and high rates of mortality are usually observed.(192)

Vaccination

Outbreaks of respiratory disease following BRSV infection usually occur in calves between 2-6-months of age and vaccination with MLV or KV BRSV vaccines has been adopted as an important preventive strategy by cattle producers and veterinarians; however, vaccination of young calves with high levels of maternal antibodies derived from colostrum can interfere with vaccine immune responses and efficacy.(265, 266) Additionally, exacerbation of BRSV

clinical disease and high mortality rates have been reported in young calves previously vaccinated with KV BRSV vaccines following natural infection with field BRSV viruses.(7, 267) Similar results were reported in human infants previously vaccinated with a formalin-inactivated HRSV vaccine that resulted in severe exacerbation of respiratory disease signs following natural exposure to the virus.(268) A type-1 hyperpersensitivity reaction initiated by vaccine priming of specific IgE (Th2 response) it is believed to have led to a severe inflammatory reaction resulting from histamine production and pulmonary edema and emphysema in infants and calves that had received a KV vaccine prior natural exposure.(157, 267, 268)

Following experimental infection with BRSV, previously vaccinated calves shed less virus in nasal secretions compared with unvaccinated controls.(269) Clearance of the virus after vaccination coincides with mucosal antibody formation (IgA), presence of cytotoxic T cells within the lungs, and serum antibody responses.(270) Serum antibody titers against BRSV following vaccination of calves can be found up to 112-123 days after.(271) Serum as well as mucosal antibody responses are influenced by age at vaccination and level of maternal antibodies in calves at the time of vaccination. Based on results from different studies, serum maternal antibody titers against BRSV as low as 32 are capable of interfering with seroconversion to BRSV vaccination in young calves.(9, 272, 273)

Both parenteral and intranasal BRSV vaccines have been commercially available since the 1980s. Most of these vaccines are multivalent combinations of BRSV with some or all of the following viruses: BHV-1, PI3, and BVDV types 1 and 2. The efficacy of administration of KV and MLV BRSV vaccines on reducing clinical disease caused by natural BRD occurrence or

experimental BRSV infection in calves has been inconsistent in the literature. Results from a recent systematic review and meta-analysis suggested that vaccination of young calves with MLV BRSV vaccines did not have a significant effect on reducing BRD-associated morbidity and mortality when comparing vaccinated and unvaccinated calves.(5) Results from previous studies suggest that it is possible that the presence of maternally derived immunity may not provide complete clinical protection at the same time that it may interfere with induction of specific mucosal (IgA) and systemic antibody responses to BRSV experimental infection or vaccination in calves.(190, 274-276) In contrast to an apparent inhibition in the development of adaptive immune (humoral or cell mediated) responses in calves vaccinated with parenteral MLV BRSV vaccines in the face of maternal antibody (IFOMA), results from other studies demonstrated that vaccination of calves IFOMA reduced the severity of clinical disease, reduced viral shedding and demonstrated some mucosal and serum antibody responses following experimental BRSV infection.(269, 270, 273, 277, 278) Despite the information obtained from different BRSV vaccination studies, it is still unclear if BRSV vaccination of calves provides clinical advantages against natural occurrence of BRSV infection and BRDC. The duration and effect of maternally-derived immunity, route, and time of vaccination with respect to potential exposure to BRSV may play an important role on efficacy of vaccination programs and protection against clinical disease in cattle.

The ability of BRSV vaccination to induce specific immune responses could be associated with vaccination efficacy and clinical protection. Results from one study suggested that parenterally MLV BRSV vaccinated calves had significantly less clinical disease, hypoxemia, virus shedding, and lung lesions on necropsy compared with unvaccinated

calves.(269) These changes were associated with greater levels of plasma IFNγ and MHC1-restricted cytotoxic T cells in MLV BRSV vaccinated calves suggesting that adequate vaccine induction of cell mediated immunity is associated with reduction of clinical disease. In contrast, results from two different studies did not find significant differences in clinical signs of disease between vaccinated and unvaccinated calves following experimental challenge with BRSV; however, vaccinated calves had higher levels of serum antibodies against BRSV and viral shedding was decreased from 3.4 to 1.2 days in this group.(279, 280) These results suggest that humoral immune responses induced by vaccination could play an important role on reduction of BRSV replication and shedding.

Induction of both local (IgA) and systemic antibody responses following vaccination of calves has been associated with clinical protection against experimental infection with BRSV.(244, 276) In this case, some investigators have suggested that IN MLV BRSV vaccines are superior to parenteral MLV BRSV vaccines to induce protective BRSV-IgA and systemic IgG responses after BRSV challenge;(9, 265, 276, 281) however, results from other studies demonstrated that parenteral MLV BRSV vaccines could effectively induce mucosal (IgA) as well as systemic specific humoral immunity.(278, 282) Results from another study suggested that exposure of the nasal mucosa to IN MLV BRSV vaccines IFOMA in calves can override the interference of maternal immunity and is effective in priming memory immune responses in young calves.(277) Several studies since then have evaluated the effect of vaccinating young calves seronegative or IFOMA with IN MLV BRSV vaccines during the first weeks of life on the developing of immune responses and clinical protection against experimental BRSV challenge as early as 2 weeks and up to 4.5 months following vaccination.(8, 265, 266, 277, 283)

The results of these studies are inconsistently demonstrate efficacy of vaccination on reducing calf morbidity and mortality after challenge. Additionally, humoral and cell mediated responses were variable among studies depending on the time of vaccination, route of administration and the presence of maternal antibodies at the time of vaccination in study calves. Based on the results of these studies an important conclusion is that the duration of mucosal BRSV humoral immunity (IgA) induced by IN MLV vaccination is short (<120 days) and it is possible that repeated exposure or vaccination of calves is necessary to maintain protective titers of BRSV IgA in the upper respiratory tract

Although the majority of studies on BRSV vaccination in calves involve MLV vaccines, results from studies evaluating KV BRSV vaccines have similarly produced inconsistent results regarding the induction of immune responses and clinical protection against experimental BRSV infection.(284-287) It is possible that literature on KV BRSV vaccines is limited by previous reports of fatal adverse reactions of calves vaccinated with KV vaccines that subsequently were exposed to field BRSV developing high levels of morbidity and mortality. The induction of a Th2 immune response with high levels of specific BRSV IgE driven by KV vaccines that results in a severe immunopathological reaction following field exposure to BRSV has been associated with this phenomenon.(267, 269, 288, 289)

Chapter 3: Statement of objectives

The general objective of this research was to evaluate mucosal and systemic BRSV antibody responses induced by different vaccination protocols using commercially available vaccines in beef cattle on clinical protection against experimental infection with BRSV. The information generated through this research will be fundamental to understand the role of maternally derived immunity and antibody responses (mucosal and systemic) elicited from vaccination on clinical protection of calves against experimental infection with BRSV to provide effective recommendations on vaccination programs for U.S. cow-calf operations to producers and veterinarians.

Specific objective 1: To perform a systematic review and meta-analysis of published literature that evaluated the effect of vaccination of dairy and beef calves under 6 months of age with commercially available BRSV vaccines on the risk ratios of becoming sick or dying following experimental infection with BRSV.

Specific objective 2: To determine if the presence of BRSV serum neutralizing antibodies at the time of initial vaccination was associated with morbidity and mortality outcomes within a systematic review and meta-analysis of published clinical trials evaluating the effect of vaccination of young calves with commercially available BRSV vaccines.

Specific objective 3: To compare the initial titers and duration of nasal BRSV-IgG1 and IgA, and serum BRSV neutralizing antibodies in beef calves born to dams that were vaccinated or not vaccinated against BRSV during the last trimester of gestation.

Specific objective 4: To determine if residual clinical protection was afforded by specific mucosal and systemic BRSV antibodies transferred from maternal colostrum to beef calves born to vaccinated or unvaccinated dams and experimentally challenged with BRSV at 3 months of age.

Specific objective 5: To determine if vaccination of beef calves with an IN MLV BRSV vaccine within 6 h of birth and before complete absorption and transfer of colostral IgG1 resulted in adequate priming and duration of nasal BRSV-IgA responses.

Specific objective 6: To determine if vaccination of beef calves with an IN MLV BRSV vaccine within 6 h of birth and before complete absorption and transfer of colostral IgG1 provided clinical advantages following experimental infection with BRSV at 3.5 months of age.

Specific objective 7: To evaluate the effect of vaccination of beef calves with a combination vaccine protocol at branding (2 months of age) and weaning (6-8 months of age) with MLV subcutaneous (SC) and MLV IN BRSV vaccines, respectively, versus single IN MLV BRSV vaccination at weaning on mucosal (IgA) and systemic antibody responses and clinical protection against simultaneous experimental challenge with BRSV and BHV-1.

Specific objective 8: To determine changes in the detection of genetic material of common bacterial pathogens of the BRDC (*Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni* and *Mycoplasma bovis*) in nasal secretion samples from weaning age beef calves immediately before and following intranasal MLV BRSV vaccination and simultaneous experimental challenge with BRSV and BHV-1.

Chapter 4: Efficacy of bovine respiratory syncytial virus (BRSV) vaccines to reduce morbidity and mortality in calves within experimental infection models: a systematic review and meta-analysis.

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David A. Martinez, Benjamin Newcomer, Thomas Passler and Manuel F. Chamorro

Abstract

Producers and veterinarians commonly use vaccination as the main strategy to reduce the incidence of bovine respiratory syncytial virus (BRSV) infection in calves; however, supportive evidence of BRSV vaccination efficacy has been inconsistent in the literature. The objective of this meta-analysis was to evaluate data from controlled studies on the efficacy of commercially available BRSV vaccines on reducing calf morbidity and mortality after experimental infection with BRSV. A systematic review and meta-analysis was performed in BRSV experimental challenge studies that reported the efficacy of commercially available modified-live virus (MLV) and inactivated BRSV vaccines on protection against calf morbidity and mortality. The studies included in the analysis were randomized, controlled, clinical trials with clear definitions of calf morbidity and mortality. Risk ratios with 95% confidence intervals and forest plots were generated. Fourteen studies including 29 trials were selected for the analysis. Commercially available MLV BRSV vaccines reduced the risk of calf mortality after experimental infection

with BRSV. Modified-live virus vaccines reduced the risk of morbidity in calves with absence of serum maternal antibodies at initial vaccination, but failed to demonstrate significant morbidity reduction when calves were vaccinated in the face of maternal immunity. Results from experimental challenge studies do not always represent the conditions of natural infection and caution should be used when making vaccine recommendations.

Introduction

Bovine respiratory syncytial virus (BRSV) is considered one of the most important viral pathogens of the bovine respiratory disease complex (BRDC) in calves.(191) A high seroprevalence of BRSV in the United States (U.S.) ranging from 60% to 80% has been reported,(189) and BRSV-associated respiratory disease outbreaks are described in dairy and beef calves.(189, 191, 290) Bovine respiratory syncytial virus most commonly affects calves under 6 months of age with moderate to high morbidity rates and low mortality rates; however, the case fatality rate can be as high as 31% in some cases.(7, 267) The severity of disease following BRSV infection depends on host immunity. Clinical signs of infection, as well as overall morbidity and mortality rates, are lower when calves have moderate to high levels of BRSV neutralizing serum antibodies, regardless if those were acquired passively through maternal colostrum or actively generated by prior BRSV infection or vaccination.(244, 277, 291) Different experimental challenge and natural infection studies have been conducted to determine the efficacy of BRSV vaccination protocols to prevent BRSV infection and disease in calves.(5, 7, 9, 265, 267, 278, 287) Results from a previous meta-analysis suggested a lack of efficacy of BRSV vaccination on the reduction of morbidity and mortality associated with bovine respiratory disease.(5) However, during the past decade, several randomized clinical trials

evaluating clinical protection afforded by calf vaccination demonstrated moderate protection after experimental infection with BRSV.(9, 265, 273, 277, 292) Results from these studies suggest that a significant reduction in the titers and duration of BRSV shedding after challenge is observed in vaccinated calves compared with non-vaccinates; however, significant and consistent reduction of respiratory clinical scores in calves is variable among published BRSV vaccination efficacy studies.(273, 278) The first objective of this study was to perform a systematic review and meta-analysis of published literature to evaluate if dairy and beef calves under 6 months of age vaccinated with commercially available BRSV vaccines had lower risk ratios of becoming sick or dying after experimental challenge with BRSV, compared with non-vaccinated control calves. The second objective of this study was to determine if the presence of BRSV serum neutralizing antibodies at the time of initial vaccination was associated with morbidity and mortality outcomes. The overarching goal of this meta-analysis was to provide BRSV calf-vaccination efficacy information to the veterinary community.

Materials and methods

This systematic review and meta-analysis was performed following PRISMA 2020 guideline recommendations. Studies in the English language that reported the efficacy of commercially available BRSV vaccines in calves undergoing experimental challenge with BRSV published in peer reviewed scientific journals were identified. The literature search, the inclusion/exclusion criteria, and data extraction was performed by all authors following an objective protocol agreed upon prior the start of the meta-analysis. The literature search was performed in March 2021 using four scientific databases with no publication date restrictions, namely PubMed, CAB, Web of Science, and Agricola using the keywords “viral” or “virus”,

"bovine" or "calf" or "cattle", "vaccine" or "immunization", and "respiratory" or "BRD", or "BRSV". After the initial search, review articles, book chapters, and duplicated articles were excluded from the analysis. When available, filters were used to exclude review articles during the search. Articles collected from the database search were included in the meta-analysis based on the following criteria: 1. The article was relevant to the study objectives; 2. The article was a randomized clinical trial and included a non-vaccinated/sham vaccinated control group; 3. The article described the use of a commercially-available BRSV-containing vaccine; 4. The article involved experimental infection with BRSV; 5. The article reported clinically relevant and well-defined outcomes (i.e., morbidity and mortality rates). Articles were excluded from the analysis if the four authors agreed they did not meet the inclusion criteria.

Relevant data for outcome analysis such as the total number of sick calves (morbidity) and the total number of dead calves (mortality) after experimental infection with BRSV was extracted from all articles and their respective trials. Only studies using commercially available BRSV-containing vaccines were used in this meta-analysis because of their relevance on providing practicing veterinarians information applicable to their clients. To evaluate the morbidity risk, the number of calves with signs of respiratory disease associated with BRSV challenge such as abnormal respiratory rate and effort, abnormal rectal temperature, nasal discharge, abnormal mentation (lethargy), the presence of cough, and the presence of abnormal lung sounds (crackles and wheezes) were noted for each trial. To evaluate mortality risk, the number of calves reported as dead following experimental infection with BRSV or humanely euthanized due to the severity of respiratory disease because of experimental infection with BRSV were noted. If calves were euthanized during any portion of the study for reasons different

from a humane endpoint associated with BRSV infection following challenge, mortality data were not included in the analysis. Data were analyzed using a commercially available meta-analysis software (CMA, Biostat, Englewood, NJ, USA). To compare the probability of an outcome in exposed (vaccinated) groups with the probability of the same outcome occurring in the non-exposed (non-vaccinated) groups, the risk ratio (RR) and 95% CI for each outcome were used as effect size. The heterogeneity among studies or trials was assessed by the Cochran Q statistic, with $P \leq 0.10$, and I^2 statistic > 50% indicating heterogeneity. A random effects model was used to compare mean effect size across treatment groups and forest plots were constructed for each meta-analysis. The forest plots generated by the software excluded trials without a significant effect on each outcome (i.e., equal morbidity and/or mortality rates in animals from exposed and non-exposed groups). Summary measures were considered significantly different between treatment groups if the 95% CI did not include 1. In some studies, the same control group was used in different trials. Publication bias was visually assessed using funnel plots of the standard error by log risk ratio.

The type of vaccine used and the presence of BRSV serum neutralizing antibodies of maternal origin at the time of initial vaccination were assessed in all studies. For studies evaluating MLV vaccines, the effect of route of administration was evaluated. To determine the effect of vaccination, the effect of type of vaccine (MLV vs. inactivated), effect of the presence or absence of BRSV antibodies at the time of initial vaccination, and effect of route of MLV vaccine administration (intranasal vs. parenteral), quantitative syntheses were performed within each outcome using a subset of studies relevant to that outcome. The final meta-analysis included separate evaluations of morbidity and mortality outcomes from vaccination in general,

vaccination with MLV vaccines, and vaccination with inactivated vaccines. The effect of maternal antibodies on morbidity and mortality outcomes was evaluated for each separate analysis.

Results

The total number of studies identified in the initial literature search was 323. Following evaluation of abstracts and complete review of articles eligible for the study, 14 studies comprising 29 different trials were selected for the meta-analysis (Figure 4.1).(5, 7, 8, 53, 244, 269, 270, 277, 278, 287, 292-296) Studies excluded from the analysis included 309 articles. The reasons for exclusion were that studies were not a randomized clinical trial, were not a peer-reviewed article, did not include commercially available vaccines, or did not include clinically relevant outcomes of morbidity and mortality such as number of animals with signs of respiratory disease after experimental challenge, number of animals dying after experimental challenge, and clinical respiratory scores. Within the 29 trials from the 14 studies, 5 evaluated inactivated vaccines, 23 evaluated MLV vaccines, and 1 evaluated an MLV vaccine followed by a booster with an inactivated vaccine (Table 4.1). While calves in some of the trials received only a single vaccine dose, calves in other studies were also administered a booster. Visual assessment of the funnel plots demonstrated an approximately symmetric inverted funnel shape distribution of the data points which is the pattern expected when publication bias is unlikely (data not shown).

Study	Vacc. Type	Route	Study calves	Maternal antibodies	Age at vaccination (days)	Booster (days)	Challenge after vacc. (days)	Follow up time (days)
Xue et al, 2010	MLV	IN	Dairy	No	5.5	NA	21	14
West et al, 1999-1	MLV	SC	Dairy	No	21	21	21	8
West et al, 1999-2	MLV	SC	Dairy	No	21	NA	21	8
West et al, 1999-3	MLV	SC	Dairy	No	21	NA	21	8
Ellis et al, 2007-1	MLV	IN	Dairy	No	42	21	21	8
Ellis et al, 2007-2	MLV	IN	Dairy	No	63	NA	21	8
Ellis et al, 2007-3	MLV	IN	Dairy	No	14	NA	8	8
Vangeel et al, 2007-1	MLV	IN	Dairy	No	21	NA	21	8
Vangeel et al, 2007-2	MLV	IN	Dairy	No	21	NA	10	8

Vangeel et al, 2007-3	MLV	IN	Dairy	No	21	NA	5	8
Vangeel et al, 2007-4	MLV	IN	Dairy	Yes	21	NA	66	8
Ellis et al, 2010-1	MLV	IN	Dairy	Yes	5.5	NA	135	8
Ellis et al, 2010-2	MLV	IN	Dairy	No	5.5	NA	135	8
Ellis et al, 2010-3	MLV	IN	Dairy	No	5.5	NA	21	8
Ellis et al, 2010-4	MLV	SC	Dairy	No	5.5	NA	21	8
Ellis et al, 2013-1	MLV	IN	Dairy	No	6	NA	49	8
Ellis et al, 2013-2	MLV	IN	Dairy	Yes	7	NA	63	8
Ellis et al, 2013-3	MLV	IN	Dairy	Yes	7	NA	105	8
Ellis et al, 2014	MLV	IN	Dairy	Yes	9	NA	77	8
Gray et al, 2019-1	MLV	IN	Dairy	No	7	NA	42	8

Gray et al, 2019-2	MLV	IN	Dairy	No	7	NA	42	8
Kolb et al, 2020	MLV	SC	Dairy	Yes	30	NA	90	8
Ellis et al, 2001-1	KV	SC	Dairy	Yes	63	21	42	8
Patel, 2004-1	KV	SC	Mix	No	14	21	126	14
Patel, 2004-2	KV	SC	Mix	No	14	21	266	14
Ellis et al, 2005	KV	SC	Dairy	No	63	20	46	8
Ellis et al, 2018-1	MLV / MLV	IN / SC	Beef	Yes	1	60	120	7
Ellis et al, 2018-2	MLV / KV	IN / SC	Beef	Yes	1	60	120	7
Sluijs et al, 2010	KV	SC	Dairy	Yes	14	NA	28	35

Table 4. 1 Studies evaluating the effect of vaccination on clinical protection against experimental infection with BRSV in calves.

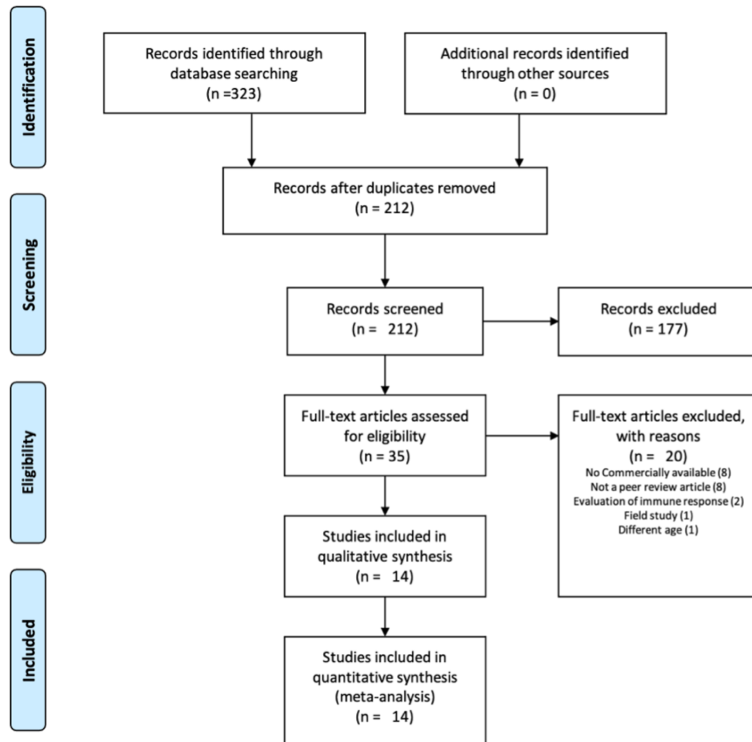

Figure 4. 1PRISMA flow diagram of studies included in the analysis.

BRSV-vaccination

The efficacy of vaccination either with MLV or inactivated BRSV vaccines on clinical protection of beef and dairy calves against experimental BRSV challenge was evaluated in 29 trials from 14 different studies (Figures 4.2 - 4.5). Of the 29 trials, 10 reported the presence of maternal antibodies at initial vaccination of study calves and 19 reported absence of maternal antibodies at initial vaccination of study calves.(5, 7, 8, 53, 244, 269, 270, 277, 278, 287, 292-296) The analysis demonstrated a 41.3% reduction in the mortality risk (RR = 0.587; 95% CI 0.436-0.792) for vaccinates, compared with controls independently of type of vaccine or the

presence or absence of maternal antibodies at initial vaccination (Figure 4.2). A 50.6% reduction of the morbidity risk (RR = 0.494; 95% CI 0.304-0.803) was demonstrated in trials in which calves had no serum neutralizing antibodies of maternal origin at initial vaccination (Figure 4.3). In trials in which calves had serum neutralizing antibodies at initial vaccination, the morbidity risk was not significantly different between vaccinates and controls.

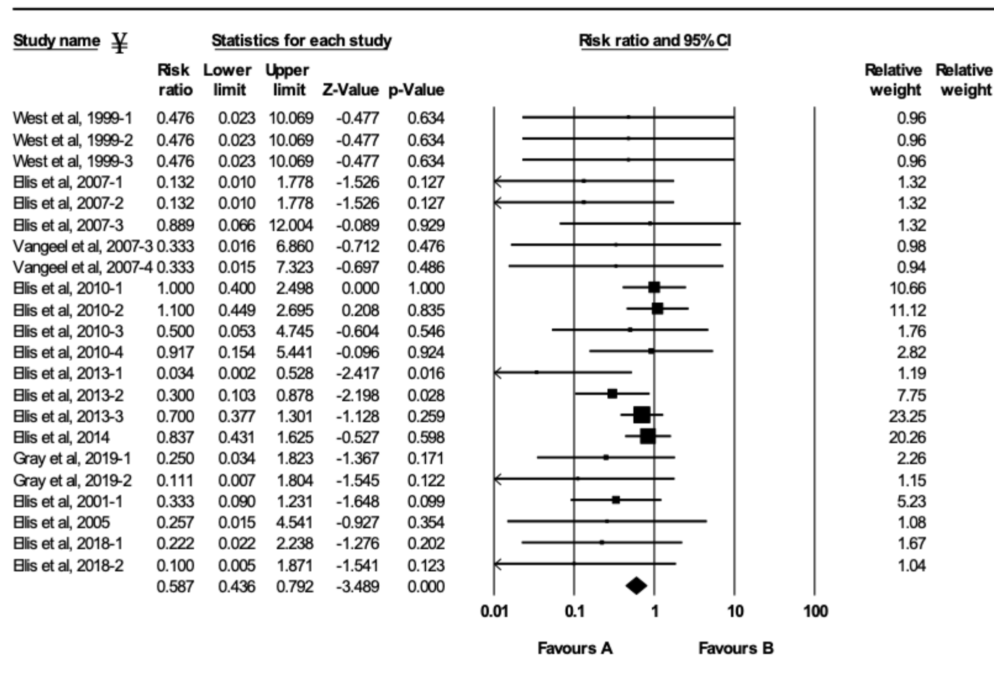

Figure 4. 2 Forest plot of mortality risk ratios from experimental BRSV challenge trials that evaluated MLV and inactivated BRSV vaccines.

¥ Only trials with a significant mortality effect between vaccinated and control calves are shown by the meta-analysis software in the forest plot

≠ Heterogeneity stats: Q-Value: 18.64, df(Q): 21 p: 0.61 I^2: 0

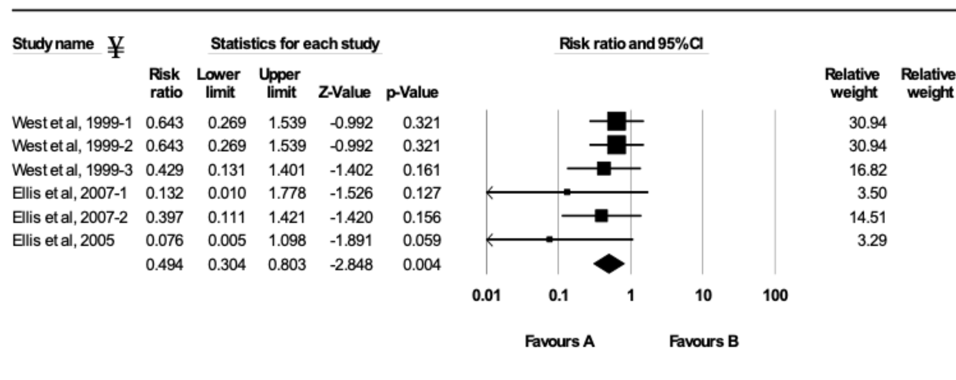

Figure 4. 3 Forest plot of morbidity risk ratios from experimental BRSV challenge trials that evaluated MLV and inactivated BRSV vaccines in seronegative calves (absence of maternal antibodies at initial vaccination).

¥ Only trials with a significant morbidity effect between vaccinated and controls calves are shown by the meta-analysis software in the forest plot

≠ Heterogeneity stats: Q-Value: 6.19, df(Q): 6 p: 0.40 I^2: 3.21

Vaccination with MLV BRSV vaccines

Ten studies comprising 23 trials evaluated the effect of MLV vaccination on clinical protection of beef and dairy calves against experimental infection with BRSV.(5, 7, 269, 270, 277, 278, 293-296) Within the 23 trials, calves from 7 trials had serum maternal antibodies at initial vaccination. In contrast, calves from 16 trials did not have serum maternal antibodies at initial vaccination. A 37.5% reduction of the mortality risk (RR = 0.625; 95% CI 0.458-0.852) for vaccinates compared with controls was demonstrated independently of the presence or absence of maternal antibodies at initial vaccination and route of administration (Figure 4.4). A 47.4% reduction of the morbidity risk (RR = 0.526; 95% CI 0.321-0.863) in vaccinates compared with controls was demonstrated in trials in which calves had no titers of maternal

antibodies at initial vaccination independently of route of administration (Figure 4.5). In trials in which calves had maternal antibodies at initial vaccination, the analysis did not demonstrate a significant reduction of the morbidity risk in vaccinates compared with controls regardless route of vaccine administration (intranasal = 4 trials vs. parenteral = 2 trials).

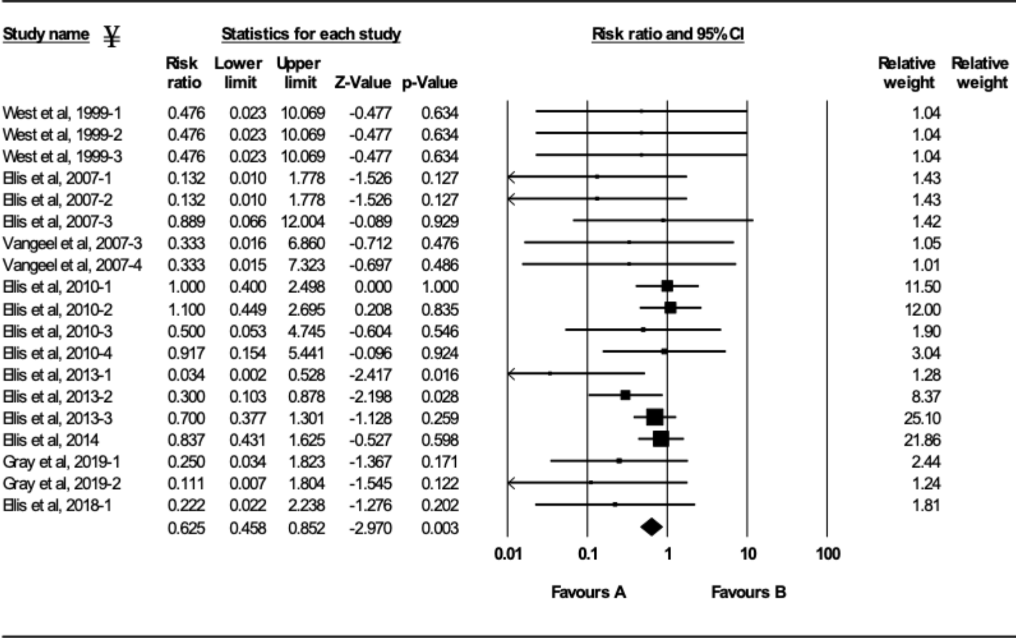

Figure 4. 4 Forest plot of mortality risk ratios from experimental BRSV challenge trials that evaluated MLV BRSV vaccines.

¥ Only trials with a significant mortality effect between vaccinated and controls calves are shown by the meta-analysis software in the forest plot

≠ Heterogeneity stats: Q-Value: 16.04, df(Q): 18 p: 0.59 I^2: 0

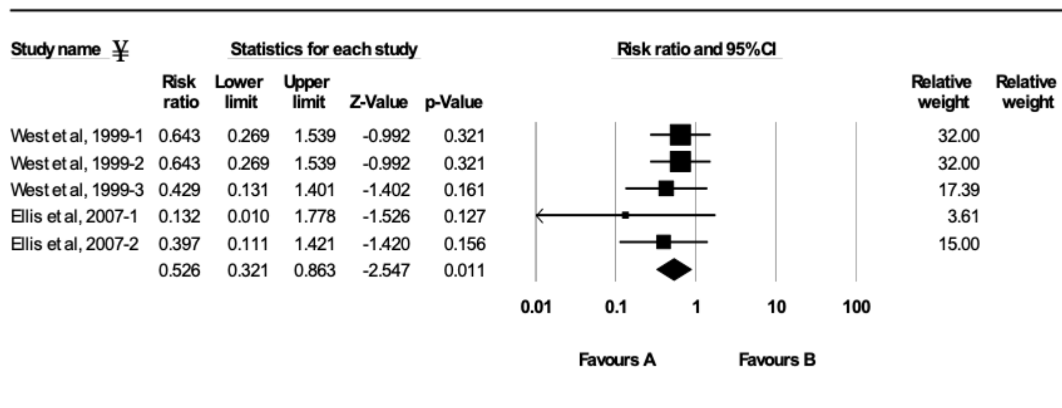

Figure 4. 5 Forest plot of morbidity risk ratios from experimental BRSV challenge trials that evaluated MLV vaccines in seronegative calves (absence of maternal antibodies at initial vaccination).

¥ Only trials with a significant morbidity effect between vaccinated and controls calves are shown by the meta-analysis software in the forest plot
≠ Heterogeneity stats: Q-Value: 3.55, df(Q): 5, p: 0.62, I^2: 0

Vaccination with inactivated-BRSV vaccines

Four studies including 5 different trials evaluated clinical protection afforded by vaccination of calves with inactivated vaccines against experimental infection with BRSV.(8, 244, 287, 292) Similar morbidity and mortality risks in vaccinates compared with controls were demonstrated by the analysis. The number of trials using inactivated BRSV vaccines and reporting the presence or absence of maternally derived immunity at initial vaccination was insufficient to evaluate the effect of maternal antibodies on morbidity and mortality outcomes following vaccination and challenge.

Discussion

This meta-analysis provides quantitative information about the efficacy of commercially available BRSV vaccines reducing calf morbidity and mortality following experimental BRSV infection. In this meta-analysis, vaccination of calves under 6 months of age with MLV BRSV vaccines demonstrated a significant reduction of the mortality risk associated with experimental BRSV infection; however, reduction of the morbidity risk was demonstrated only for calves that did not have serum neutralizing antibodies at the time of vaccination. Vaccination of calves under 6 months of age with inactivated BRSV vaccines did not result in significant reduction of morbidity or mortality risks associated with experimental BRSV infection. Our results contrast with the results of a previous meta-analysis.(5) Theurer et al. (2015) reported that no significant reduction of the morbidity and mortality risks after experimental infection with BRSV in calves previously vaccinated with MLV BRSV vaccines. The authors of that study commented that a greater study heterogeneity in studies involving MLV vaccines could have limited their ability to detect a significant effect on morbidity and mortality among MLV-vaccinated and control calves. Interestingly, results from that same study (5) demonstrated a significant reduction of calf mortality after experimental BRSV challenge in calves vaccinated with an inactivated BRSV vaccine. It is possible that lack of power and greater chance for making a type II error resulting from a low number of trials using inactivated vaccines in this meta-analysis prevented the observation of significant differences on calf morbidity and mortality in studies involving inactivated vaccines. Additionally, results from previous studies suggest that incomplete and short-lived humoral and cell-mediated immune memory responses induced by inactivated vaccines could be associated with their lack of significant effects on calf morbidity and mortality in BRSV experimental challenge trials.(293, 297) Although BRSV is a single piece of the puzzle in the natural occurrence of the bovine respiratory disease complex (BRDC), its seroprevalence

in U.S. cattle populations is high, and case-fatality rates of acutely infected cattle can reach 31% in some cases.(267) Therefore, the efficacy of MLV BRSV vaccines on reducing calf mortality following experimental BRSV infection provides evidence of the importance of the BRSV component in vaccination programs aimed to control the natural occurrence of BRDC in cattle.

In studies where the effect of vaccination was evaluated in calves devoid of BRSV-specific serum maternal antibodies, the lack of maternal immunity was associated with reduction of calf morbidity after experimental BRSV infection in vaccinates compared with controls. It is possible that the absence of maternal interference favored the induction of complete immune responses to vaccination and prevented clinical disease in vaccinates. In contrast, in studies where the effect of vaccination was evaluated in calves with BRSV-specific serum maternal antibodies, the presence of maternal immunity was not associated with significant reduction of calf morbidity in vaccinates. It is possible that the presence of serum maternal antibodies similarly prevented clinical disease after experimental BRSV infection in vaccinated and control calves in these studies. Results from previous studies have demonstrated that the presence of maternal immunity suppress local and systemic antibody responses following vaccination and provide clinical protection against experimental infection with respiratory viruses in calves.(244, 265, 270, 294-296, 298)

The most important limitation of this meta-analysis is that it was restricted to evaluation of vaccination efficacy in experimental BRSV challenge studies. The extrapolation of vaccine efficacy data from experimental challenge studies to field conditions requires caution; however, randomized, controlled vaccination efficacy studies under natural disease occurrence conditions

do not exist in the literature, specifically with respect to BRSV. Several reasons, including the need of a considerable sample size of the target population, the need of a control/unvaccinated group (unlikely in client-owned cattle where the numbers might be adequate), the poly-microbial nature in the etiology of the BRDC, and funding limitations could explain the scarcity of these type of studies. Although vaccination efficacy analysis from experimental challenge studies might not be ideal, it provides meaningful information for practicing veterinarians in the decision-making and vaccine recommendation process. The BRDC continues to be the most economically important disease affecting cattle operations in the U.S., and BRSV is as an important etiologic agent of the complex. Therefore, experimental challenge BRSV vaccination-efficacy studies might be the only alternative for veterinarians to make evidence-based vaccination recommendations for the prevention of BRSV infection in cattle. Other limitations included the limited number of studies evaluating inactivated-BRSV vaccines and the use of the same control group in different trials (from the same study) within the same study. It is possible that some experimental challenge studies using inactivated BRSV vaccines were excluded from this analysis because of failure to meet the selection criteria. In some of these studies, exacerbation of BRSV infection and clinical disease associated with a Th2-driven immune response (BRSV-specific IgE and histamine production) was reported in calves previously vaccinated with inactivated BRSV vaccines.(7, 267, 296) Other inactivated vaccine studies evaluating recombinant protein/subunit vaccines were not included in the analysis because these types of vaccines are not commercially available. In order to include a greater number of trials for this meta-analysis the control group for each study was re-used within the analysis of data.

Conclusion

Results from this meta-analysis suggest that MLV BRSV vaccination reduces calf mortality following experimental BRSV infection. Additionally, vaccination of seronegative calves with MLV BRSV vaccines reduced calf morbidity and mortality following experimental BRSV infection. Based on this study's findings, we conclude that vaccination of calves with failure in the transfer of passive immunity, colostrum-deprived, or with lack of passive BRSV antibodies is important for prevention of clinical disease associated with BRSV infection; however, veterinarians need to use caution when recommending vaccination protocols against BRDC based on results from experimental viral infection studies.

Chapter 5: The titers, duration, and residual clinical protection of passively-transferred nasal and serum antibodies are similar among beef calves that nursed colostrum from vaccinated or unvaccinated dams and were experimentally challenged with bovine respiratory syncytial virus (BRSV) at 3 months of age.

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David A. Martínez, Manuel F. Chamorro, Thomas Passler, Laura Huber, Paul H. Walz, Merrilee Thoresen, Gage Raithel, Scott Silvis, Ricardo Stockler, Amelia R. Woolums,

Abstract

Objectives

To compare initial titers, duration, and residual clinical protection of passively-transferred bovine respiratory syncytial virus (BRSV) nasal immunoglobulin G-1 (IgG1) and immunoglobulin A (IgA), and serum neutralizing (SN) antibodies.

Animals

Forty 3-month-old beef steers born either to unvaccinated or vaccinated cows

Procedures

During the last trimester of gestation, cows were randomly assigned to either vaccinated or unvaccinated groups. After calving, group NO-VACC (n=20) included calves that nursed colostrum from unvaccinated dams and group VACC (n=20) included calves that nursed colostrum from dams vaccinated with 2 doses of an inactivated BRSV-vaccine. At 3 months of age, calves were challenged with BRSV. Following challenge, respiratory signs were scored. Nasal BRSV IgG1 and IgA and SN antibodies were compared before and after challenge. The presence of BRSV in nasal secretions was evaluated by reverse transcription (RT)-PCR.

Results

Respiratory scores after BRSV challenge were similar between treatment groups. Nasal BRSV IgG1 and SN antibodies were significantly greater in VACC calves at 48 hours of life; however, by 3 months of age, titers had decayed in both groups. Nasal BRSV IgA titers were minimal following colostrum intake and before BRSV challenge and increased in both groups after challenge. Group NO-VACC had a significantly greater probability of shedding BRSV compared with VACC calves.

Clinical Relevance

At 3 months of age, titers of passively-transferred BRSV antibodies in VACC and NO-VACC calves had decayed to non-protective levels. Calves born to vaccinated dams had a decreased probability of BRSV shedding; however, this was not related to differences in SN or nasal BRSV antibody titers.

Introduction

Bovine respiratory syncytial virus (BRSV) plays an important etiological role for respiratory disease in calves under 6 months of age and can be an important contributor to pre-

weaning beef calf pneumonia in United States (U.S.) cow-calf operations.(7, 191) Pre-weaning or "summer" beef calf pneumonia usually occurs in calves between 2 and 4 months of age.(14, 165) The vast majority of studies describing BRSV-associated calf morbidity and mortality in North America involve dairy calves with a minimal number of studies in beef calves.(265, 269, 273, 277, 278, 281) However, high morbidity and mortality rates due to respiratory disease resulting from BRSV infection in beef calves have been reported in European countries.(5, 7, 267) Vaccination of calves between 1 and 30 days of age has become a standard strategy among producers and veterinarians to prevent calf morbidity and mortality associated with BRSV infection; however, successful reduction of clinical signs of disease following calf vaccination has been inconsistent in studies of naturally occurring and experimentally induced BRSV infection.(5, 7, 267, 273, 278) The presence of colostrum-derived serum antibodies in young calves may suppress respiratory tract and circulatory antibody responses following experimental infection or vaccination and could explain the inconsistencies observed with vaccination efficacy.(244, 276) Despite interfering with vaccine-induced local (respiratory tract) and systemic humoral responses, moderate to low levels of serum neutralizing (SN) antibodies derived from colostrum protect young calves against clinical disease following acute infection with respiratory viruses such as bovine viral diarrhea 1 and 2 (BVDV 1 - BVDV 2), bovine herpesvirus 1 (BHV-1) and BRSV.(295, 299, 300) Cell mediated immune responses in previously vaccinated calves may also contribute to protection,(301, 302) but these are less frequently reported.

Immunoglobulin G-1 (IgG1) is the most prevalent immunoglobulin in colostrum and is preferentially transferred to the respiratory tract of calves following colostrum intake.(258)

Results from one study suggested that colostrum-derived IgG1 present in the upper respiratory tract of young lambs can prevent parainfluenza 3-virus replication.(303) In contrast to IgG1, the concentration of immunoglobulin A (IgA) in colostrum is minimal and its transfer to the respiratory tract of calves as well as its role in clinical protection against acute viral infection has not been described.(304) It is possible that high levels of BRSV-IgG1 transferred from colostrum to the respiratory tract reduces BRSV infection and clinical disease in young calves; however, the duration of colostrum-derived nasal IgG1 and IgA and their role on clinical protection of calves against BRSV infection has not been described. The first objective of this study was to compare the initial titers and duration of nasal BRSV-IgG1 and IgA and serum neutralizing antibodies in calves born to vaccinated or unvaccinated dams. The second objective of this study was to determine if residual clinical protection was afforded by passively-transferred BRSV antibodies. We hypothesized that vaccination of pregnant dams would result in greater transfer of colostrum-derived BRSV antibodies to their calves. Additionally, we hypothesized that a greater initial titer of colostrum-derived BRSV antibodies would improve the residual clinical protection afforded by maternal immunity following experimental challenge with BRSV at 3 months of age.

Materials and methods

Experimental design

The Auburn University Institutional Animal Care and Use Committee PRN # 2019-3530 reviewed and approved all animal protocols within this study. Cows from a single 175-head cow-

calf herd were enrolled in this randomized controlled clinical trial during pregnancy diagnosis in the summer of 2019. At approximately 6.5 to 7.5 months of gestation, cows were stratified by age and randomly assigned to two different treatment groups. The unvaccinated group received 5 mL of 0.9% phosphate buffered saline (Veltivex™® Dechra Veterinary Products, Overland Park, KS, USA) subcutaneously 21 days apart. The vaccinated group received 2 doses of a multivalent inactivated-virus vaccine (Traingle10®; Boehringer Ingelheim Animal Health USA Inc; Duluth, GA, USA) containing BRSV 21 days apart according to manufacturer's recommendations. Approximately 1 month before calving, all cows were moved into a single pasture and monitored for parturition by on-farm personnel blinded to treatment allocation.

After calving, all calves nursed colostrum naturally from their respective dams and without assistance. By 24 hours of age, all male calves were weighed, identified [individual radio frequency electronic tag and regular ear tag], and castrated. Once all calves had been born, a random sample of 20 calves each, born to vaccinated dams [VACC group (n=20)] or unvaccinated dams [NO-VACC group (n=20)] were selected for the experimental portion of the study. The study calves remained with their dams in the same pasture until early weaning. One month prior to weaning, creep feeders were introduced to the pasture to allow calves to familiarize with concentrate feed. At 3 months of age, all calves were abruptly weaned and transported 200 miles to the North Auburn BVDV research unit for the BRSV challenge portion of the study. At arrival to the unit, calves were placed in a single pasture with ad-libitum access to fresh hay, water, and concentrate feed. Five days following arrival, all calves were challenged with BRSV by intranasal nebulization as described below.

Blood samples from dams were collected before initial vaccination and at calving for virus neutralization antibody testing. At calving, maternal colostrum samples from each cow were indirectly assessed for IgG concentration by Brix refractometry (MA871 Digital BRIX refractometer; Milwaukee instruments; Rocky Mount, NC). Transfer of passive immunity was also evaluated indirectly in all calves by serum Brix refractometry at 48 hours of age. Blood and nasal secretion samples from calves were collected before and after BRSV challenge for antibody and virological assays.

BRSV challenge

On Day 0, each calf was challenged with 7 ml of a lung wash inoculum that had been expanded once in Marvin Darby bovine kidney (MDBK) cells and contained 1 x 10^4 $TCID_{50}$ of BRSV strain GA-1/ mL by individual intranasal nebulization using an electronic nebulizer (Pulmo-Aide® compressor nebulizer system, DeVilbiss Health Care; Washington, NY, USA) connected to a facemask (Era® Equine Mask, BIOMEDTECH, Melbourne, VIC, USA).

Clinical evaluation and sample collection

Following arrival at the BVDV unit and before experimental challenge with BRSV, on-farm personnel blinded to treatment allocation observed calves daily and recorded clinical signs of disease (i.e., cough, nasal discharge, diarrhea, etc.) and treatments in individual-calf notebook sheets. After BRSV challenge, clinical evaluation and scoring of calves was performed by a single veterinarian blinded to treatment group allocation on days 0, 4, 6, 8, 10, 14, 21, and 28

relative to BRSV challenge. Blood samples were collected at 48 hours and 30 days of life, and on Days 0 and 28 after challenge for BRSV serum neutralization assays. Nasal secretion samples were collected at 48 hours and 30 days of life and on Days 0, 4, 21, and 28 for BRSV IgG1 and IgA determination. Additional nasal secretion samples were collected on days 0, 4, 6, 8, 10, 14, 21, and 28 to determine the presence of BRSV by reverse transcription (RT)-PCR. Samples from each calf were labeled such that treatment allocation remained masked from personnel processing samples and performing the assays.

On sampling days, clinical signs such as depression, rectal temperature, respiratory rate, cough, and nasal discharge were evaluated and a total respiratory score was assigned to each calf as previously described.(305) Clinical signs were scored in a scale of 0 to 3, where 0 was considered normal or absent of abnormalities and 3 was the most abnormal finding. Depression was scored from 0 (bright alert responsive) to 3 (obtunded, recumbent, non-responsive), rectal temperature was scored from 0 (37.8-38.3°C) to 3 (> 39.4°C), respiratory rate was scored from 0 (respiratory rate < 30 rpm) to 3 (respiratory rate > 100 rpm), cough was scored from 0 (none) to 3 (repeated spontaneous cough), and nasal discharge was scored from 0 (none or serous discharge) to 3 (purulent bilateral discharge). The sum of individual scores including rectal temperature, depression, respiratory rate, nasal discharge, and cough determined the presence of mild, moderate, or severe respiratory disease.(305) Briefly, mild respiratory disease was determined by a sum of individual scores between 0-5, moderate respiratory disease was determined by a sum of scores between 6-10, and severe respiratory disease when the sum of scores was > 10. In addition to clinical evaluation, individual body weights were obtained at birth, on Day 0 (BRSV challenge), and days 14, and 28 after BRSV challenge using a portable

livestock electric scale (Livestock Platform Scale® Brecknell, Fairmont, MN, USA) that was calibrated prior to and after each weighing.

BRSV neutralizing antibodies in serum

A virus neutralization assay for the detection of serum anti-BRSV antibodies was performed as previously described.(282) Serum samples were thawed and heat inactivated in a water bath at 55°C for 30 minutes, then serial 2-fold dilutions (1:10 to 1:1,000) were made in 96-microwell flat-bottom plates, then 500 µL of 100 TCID50 BRSV suspended in minimum essential medium were added to all wells. For each dilution, 3 microwells were inoculated with equal volumes of virus culture media. Following incubation at 37°C in 5% CO_2 for 1 hour, MBDK cell cultures were inoculated with minimum essential medium that included 7% bovine serum and an antibiotic/antimycotic solution containing streptomycin, penicillin, and amphotericin B. The plates were then incubated for up to 2 weeks and monitored daily for the presence of cytopathic effect by microscopic evaluation. Antibody titers were then reported as the inverse of the lowest dilution of serum required to inhibit all cytopathic effect and were Log_2 transformed for statistical analysis.

Determination of anti-BRSV IgG1 and IgA in nasal secretions

Bovine respiratory syncytial viral particles were inactivated with 2µM binary ethyleneimine, neutralized with sodium thiosulfate, and diluted 1:800 in carbonate bicarbonate buffer (pH, 9.5). The resulting solution was used to coat microwells of 96-well polystyrene

plates. After coating, the plates were incubated overnight at 4°C and washed 3 times with PBS containing 0.05% polysorbate 20 (Tween®20, Sigma-Aldrich, St. Louis, MO, USA). After washing, 200 µL of PBSS solution containing 5% sheep serum albumin (Sheep serum albumin; Sigma-Aldrich; St. Louis, MO, USA) were added to each well for blocking, the plates were incubated at 37 ºC for 1 hour, then washed 3 times.

Vials containing nasal secretion samples were thawed and vortexed. Each sample was initially diluted 1:1 in Pluronic F127 and then diluted 1:100 in polysorbate 20. From this dilution, serial 2-fold dilutions were prepared up to 1:200 for IgG1 and up to 1:1600 for IgA and each dilution was analyzed in triplicate (i.e., each sample dilution was added to 3 wells). If the coefficient of variation among the 3 values was > 20%, the outlier value was removed, and the mean value of the 2 remaining samples was used in the calculation of the antibody titer. Samples with an optical density value that was too high to be measured accurately were tested again at a higher dilution. Samples with an optical density value that was too low to be measured accurately were tested again at a lower dilution with the lowest dilution tested being 1:25 for IgA and 1:100 for IgG1. In addition to the samples, each plate had 4 microwells containing the following: positive control, which was a nasal secretion sample from a known BRSV antibody positive calf diluted 1:100 in polysorbate 20; negative control, which was low-IgG fetal bovine serum (FBS, Sigma-Aldrich; St. Louis, MO, USA) diluted 1:100 in polysorbate 20; and blank, which was polysorbate 20 alone. For BRSV-specific IgA, horseradish peroxidase-conjugated rabbit anti-bovine IgA (Rabbit anti-bovine IgA, Bio-Rad, Hercules, CA, USA) diluted 1:500 in an ELISA wash buffer (PBS + 0.05% TWEEN 20) and 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) substrate solution (ABTS, Sigma-Aldrich, St. Louis,

MO, USA) were added to each well. For BRSV IgG1, horseradish peroxidase (HRP)-conjugated sheep anti-bovine IgG1 (Sheep anti-bovine IgG1 HRP; Bio-Rad, Hercules, CA, USA) diluted 1:7500 in an ELISA wash buffer (PBS + 0.05% TWEEN 20) and o-phenylenediamine dihydrochloride (OPD) substrate were added to each well. All plates were read by a plate reader set at a wavelength of 405 nm. Wells positive for anti-BRSV IgA and IgG1 yielded a green (IgA) and yellow (IgG1) product when the bound peroxidase-conjugated rabbit anti-bovine IgA and sheep anti-bovine IgG1 reacted with the ABTS and OPD substrates, respectively. Immunoglobulin A and IgG1 titers were reported as the inverse of last dilution that was ≥ 2 times the mean optical density value of the negative control.

Determination of BRSV RNA in nasal secretions

Real time RT-PCR was performed in nasal secretion samples as previously described.(306) Briefly, nasal secretion sample aliquots were subjected to RNA extraction using RNAzol® (Sigma-Aldrich, St. Louis, MO, USA) following manufacturers recommendations. Once extracted, the RNA templates were reverse transcribed and amplified with qScript™ XLT One-Step RT-qPCR ToughMix (qScript™® Sigma-Aldrich, St. Louis, MO, USA) using BRSV specific primers and probes.(306) All reactions were performed in a Light Cycler 480® II (Roche, UK) and results were analyzed by Light Cycler 480® SW 1.5 software (SW 1.5 software, Roche, UK).

Statistical analysis

Data were analyzed using statistical software (RStudio version 1.4.1717, Posit^{TM}). The normality of the data was assessed using the Shapiro–Wilk test and examination of the residuals. Data were analyzed using generalized mixed-effects models with animal ID as the random effect, immunoglobulin titers, virus neutralization titers, rectal temperatures, and body weights as dependent variables and vaccination status and experiment time as independent variables. Post-hoc familywise comparisons were performed using Tukey-Kramer with Bonferroni correction. Kaplan–Meier curves were generated to display BRSV shedding via nasal secretions over time for NO-VACC versus VACC calves. For non-parametric variables and proportions (i.e., brix values, clinical scores), group comparisons were performed using the Fisher's exact test, Chi-square (categorical predictor variables, 2 groups), Kruskal-Wallis (categorical predictor variables, >2 groups), and Wilcoxon Rank-Sum test (to compare medians between 2 independent populations). For all analyses, significance was set at p-value < 0.05.

Results

Transfer of passive immunity and clinical outcomes

The median of maternal colostrum Brix at calving was greater in unvaccinated cows (28.1%) compared with vaccinated cows (26.4%); however, this difference was not statistically significant (P = 0.28). Similarly, the median serum Brix reading at 48 hours of life was greater in calves that nursed colostrum from unvaccinated dams (NO-VACC = 10.9%) compared with

calves that nursed colostrum from vaccinated dams (VACC = 10.4%); however, this difference was not statistically significant (P = 0.24).

Clinical signs of disease were not observed in calves during the pre-weaning period and before BRSV challenge. Following challenge, one group-VACC calf was found dead in the pasture on day 4. Necropsy and histopathologic evaluation of this calf revealed free gas bloat as the cause of death but no respiratory tract lesions. Signs of respiratory disease such as tachypnea, cough, nasal discharge, and depression were not different between VACC and NO-VACC calves after challenge (Table 5.1). The total respiratory score after BRSV challenge was mild and no evidence of statistical significance was detected between treatment groups (Figure 5.1). The proportion of calves that developed fever (rectal temperature > 39.7 °C) after experimental challenge with BRSV was numerically greater in group NO-VACC (20/20; 100%) compared with group VACC (15/20; 78%); however, this difference was not statistically significant (P = 0.1).

	NO-VACC	**VACC**	**p-value**	**SMD**
N	20	20		
Body weight (Kg; median [IQR])	112.72 [71.58, 124.74]	110.00 [52.23, 123.72]	0.77	0.04
Rectal temperature (°C; median[IQR])	38.9 [38.5, 39.3]	38.4 [38.4, 39.3]	0.90	0.08

Rectal temperature score (%; 0-3) (0 = 37.8-38.3°C; 3 = >39.4°C)				
0	30 (18.8)	31 (20.3)	0.79	0.12
1	39 24.4)	37 (24.2)		
2	63 (39.4)	53 (34.6)		
3	28 (17.5)	32 (20.9)		
Depression Score (%; 0-2) (0 = bright, responsive; 3 = recumbent, non- responsive)				
0	138 (86.2)	138 (90.2)	0.40	0.15
1	21 (13.1)	15 (9.8)		
2	1 (0.6)	0 (0.0)		
Cough Score (%; 0-3) (0 = none; 3 = repeated spontaneous cough)				
0	160 (100.0)	158 (98.8)	0.48	0.16
3	0 (0.0)	2 (1.2)		
Nasal Secretion (%; 0-3) (0 = none or serous; 3 = purulent bilateral)				
0	127 (79.4)	126 (82.4)	0.20	0.25
1	20 (12.5)	21 (13.7)		

2	11 (6.9)	3 (2.0)		
3	2 (1.2)	3 (2.0)		
Clinical Scores (median [IQR])	2.00 [1.00, 3.00]	2.00 [1.00, 3.00]	0.36	0.12

Table 5. 1 Descriptive statistics of body weights (BW) and clinical scores following experimental challenge with BRSV of calves from a single herd that nursed colostrum from dams vaccinated with 2 doses of an inactivated bovine respiratory syncytial virus (BRSV) vaccine (VACC group) versus calves that nursed colostrum from unvaccinated dams (NO-VACC group). Data were analyzed using generalized mixed-effects models. Data reported as number and percentage of calves unless otherwise noted. SMD represents standardized median difference.

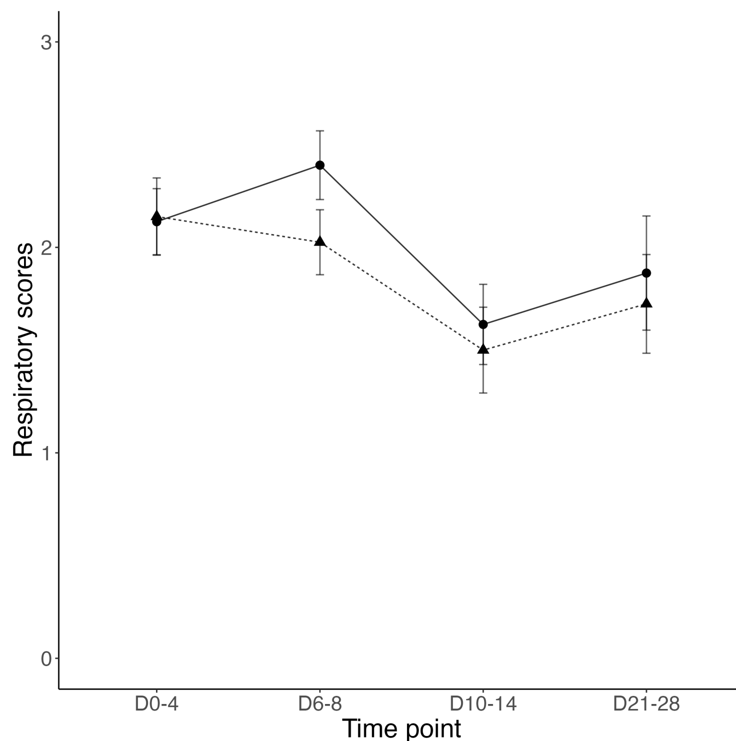

Figure 5. 1 Mean (± SEM) total respiratory scores during days 0 to 4, 6 to 8, 10 to 14, and 21 to 28 after experimental bovine respiratory syncytial virus (BRSV) challenge of calves from a single herd that nursed colostrum from dams vaccinated with 2 doses of an inactivated BRSV vaccine (VACC group; n = 20; dashed line and triangles) versus calves that nursed colostrum from unvaccinated dams (NO-VACC group; n=20 [controls]; solid line and circles). For each group and time point, the circle or triangle represents the mean, and the whiskers represent the

SEM. A line for each group connects the group's mean respiratory score (on a scale[23] from 0 [clinically normal] to 3 [most abnormal findings]) progression throughout the study. Data were analyzed using generalized mixed-effects models. No statistically significant differences were observed between time points within participants of each group or between groups.

There was a significant effect of time on the mean individual body weights within treatment groups as all calves gained weight from birth to weaning ($P < 0.01$ for all time-point comparisons within each group); however, no evidence of statistical significance was detected in the mean ± SEM individual body weights between groups throughout the study period ($P > 0.99$, for all comparisons between groups). For VACC and NO-VACC calves, the mean average daily gain (ADG) ± SEM from birth to weaning (0.75 ± 0.03 kg/d vs. 0.71 ± 0.03 kg/d, respectively) was significantly greater ($P = 0.01$) compared with the mean ± SEM ADG from challenge day to the end of the study (0.35 kg/d ± 0.03 vs. 0.31 kg/d ± 0.07, respectively; however, this difference was not statistically significant between treatment groups ($P > 0.5$).

BRSV neutralizing antibodies in serum

The mean ± SEM Log2 BRSV serum neutralizing antibody titer before vaccination was not significantly different ($P = 0.07$) between vaccinated and unvaccinated cows (4.5 ± 0.39 vs. 3.5 ± 0.55, respectively). At calving, the mean ± SEM Log2 BRSV serum neutralizing antibody titer was significantly greater ($P = 0.04$) in vaccinated cows compared with unvaccinated cows (5.15 ± 0.59 vs. 3.05 ± 0.49, respectively). At 48 hours of life, the mean Log2 BRSV serum antibody titer was significantly higher in VACC calves compared with NO-VACC calves. After the 48-h time point, serum neutralizing antibodies decreased within groups until the end of the study (Figure 5.2); The absolute mean of Log2 serum neutralizing antibody titers were greater in

VACC calves at 30 days of life, Day 0 (Challenge), and Day 28 compared with NO-VACC calves; however, these differences were not statistically significant at any time point between treatment groups.

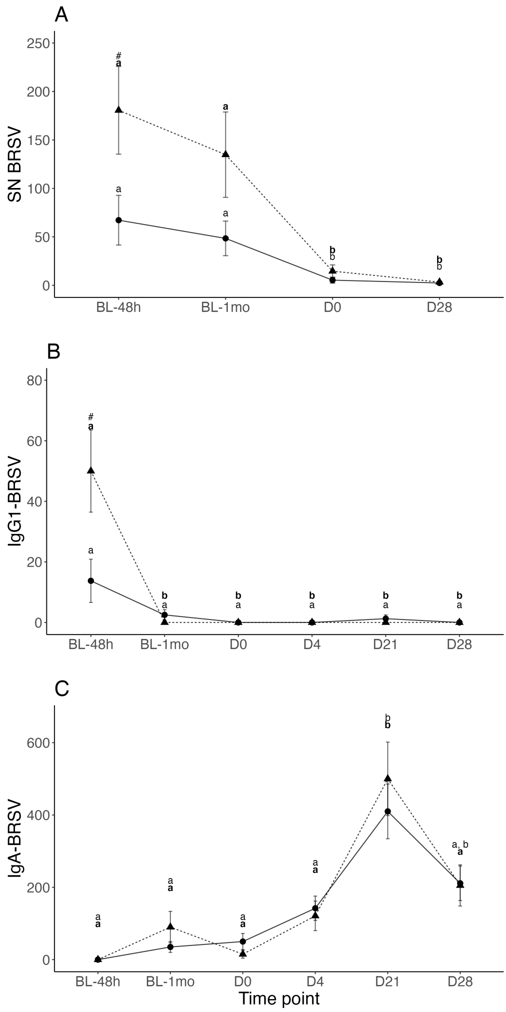

Figure 5. 2 Mean (±SEM) Log_2-Transfored serum neutralizing antibody titer (**A**), nasal secretion immunoglobulin G-1 (IgG1) bovine respiratory syncytial virus (BRSV) antibody titer (**B**), and nasal secretion immunoglobulin A (IgA)-BRSV antibody titer (**C**) for calves in the VACC (dashed line, triangles) versus NO-VACC (solid line, circles) groups described in Figure 5.1 at baseline 48 hours after birth (BL- 48h), baseline 1 month after birth (BL-1mo), and days 0 [challenge day], 21, and 28. Data were analyzed using Generalized mixed-effects models. Number signs indicate results differed significantly ($P < 0.05$) between groups; distinct letters represent significant ($P < 0.05$) difference between time-points within participants of each group

(vaccinated in bold). Familywise multi comparisons were performed using Tukey-Kramer with Bonferroni correction.

BRSV-IgG1 and IgA titers in nasal secretions

At 48 hours of life, the mean nasal BRSV-IgG1 titer was significantly greater in VACC calves compared with NO-VACC calves (Table 5.2); however, after day 30 of life, BRSV-IgG1 titers were virtually absent in nasal secretion samples from calves in both groups for the remainder of the study period (Figure 5.2). In contrast, negative or low mean nasal BRSV-IgA titers were detected in VACC and NO-VACC calves at 48 hours and 30 days of life, and the day of challenge. Nasal BRSV-IgA titers progressively increased on days 4, 21, and 28 after BRSV challenge in calves from both groups peaking at Day 21; however, statistically significant differences between groups were not observed at any time point.

Test and time point	**NO-VACC**	**VACC**	**P-value**
BRSV SN (mean Log2 ± SEM)			
48 h of life	4.7 +/- 0.5	6.2 +/- 0.5	0.04
30 d of life	4.2 +/- 0.5	5.3 +/- 0.6	0.23
Day 0 (Challenge day)	1.3 +/- 0.4	2.4 +/- 0.4	1.00
Day 28	1.1 +/- 0.1	1.4 +/- 0.2	1.00
BRSV nasal IgG1 (mean ± SEM)			
48 h of life	14 +/- 7.1	50 +/- 13	0.01
30 d of life	2.5 +/- 1.7	0	1.00

Day 0 (Challenge day)	0	0	1.00
Day 4	0	0	1.00
Day 21	1.2 +/- 1.2	0	1.00
Day 28	0	0	1.00
BRSV nasal IgA (mean ± SEM))			
48 h of life	0	0	1.00
30 d of life	35 +/- 15	90 +/- 43	0.10
Day 0 (Challenge day)	50 +/- 22	0	1.00
Day 4	121 +/- 41	142 +/- 34	1.00
Day 21	465 +/- 96	410 +/- 77	0.97
Day 28	217 +/- 59	211 +/- 48	1.00

Table 5. 2 Mean (± SEM) serum neutralizing (SN), and nasal immunoglobulin G-1 (IgG1) and immunoglobulin A (IgA), bovine respiratory syncytial virus (BRSV) antibody titers at base line (48 h and 30 d of life) and after experimental challenge with BRSV (Days 0 through 28) of calves from a single herd that nursed colostrum from dams vaccinated with 2 doses of an inactivated bovine respiratory syncytial virus (BRSV) vaccine (VACC group; n=20) versus calves that nursed colostrum from unvaccinated dams (NO-VACC group; n=20).

BRSV RT-PCR in nasal secretions

Following BRSV challenge, a significantly greater (P = 0.01) proportion of NO-VACC calves (4/20; 20%) were confirmed to be positive for BRSV by RT-PCR in nasal secretion samples compared with the proportion of VACC calves (1/20; 5%). The median number of days on which nasal secretions were positive for BRSV RT-PCR was 2 days for NO-VACC calves and 1 day for VACC calves. The risk of BRSV shedding based on RT-PCR results in nasal

secretion samples after challenge was significantly lower ($P < 0.01$) in VACC calves compared with NO-VACC calves (Figure 5.3).

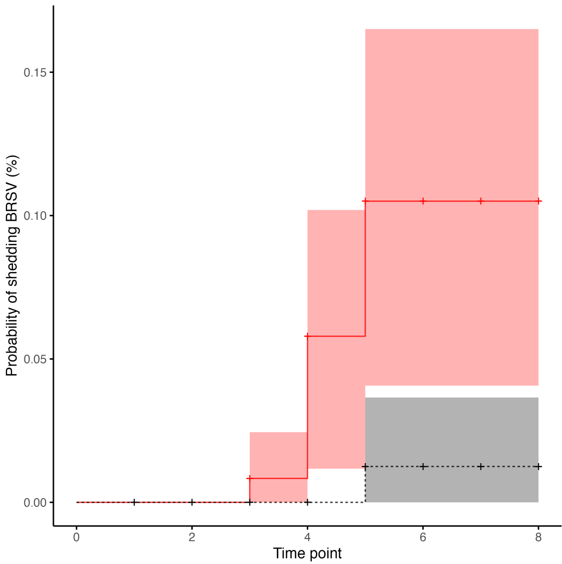

Figure 5. 3 Kaplan Meier curve of the cumulative probability of shedding bovine respiratory syncytial virus (BRSV) (detected with reverse transcription PCR assay) for calves in the VACC (dashed line) versus NO VACC (solid line) groups described in Figure 5.1 on time intervals 0 to 8, in which each time interval is representing days 0 to 2, 2 to 4, 4 to 6, 6 to 8, 8 to 10, 10 to 14, 14 to 21, and 21 to 28 after BRSV challenge. Tick marks represent the end of each time period, each step represents detection events of BRSV shedding, and shading represents the respective 95% CI for the probability of shedding BRSV by calves in the VACC (red) versus NO VACC (gray) groups.

Discussion

Maternally derived immunity against bovine respiratory viruses protects young calves against acute viral infection and disease. In the case of BRSV, results from a previous experimental BRSV infection study demonstrated reduction of clinical disease in neonatal calves with maternally derived immunity.(299) Adequate colostrum immunoglobulin G (IgG)

concentration and transfer is critical to provide effective and prolonged protection. In this study, the median Brix of colostrum in dams at calving (unvaccinated = 26.4% and vaccinated = 28.1%) was adequate and consistent with ideal IgG colostrum concentrations for beef cows.(307) Similarly, the median serum Brix of calves at 48 hours of life (NO-VACC = 10.9% and VACC = 10.6%) was adequate and consistent with what is considered excellent transfer of passive immunity in beef calves.(308) Results from previous studies(272, 309) have demonstrated that the greater the initial virus-specific serum antibody titer transferred from colostrum is, the longer the duration of specific immunity in calves lasts. Other studies,(9, 310) suggest that vaccination of cows during the last trimester of gestation with inactivated-virus vaccines results in greater deposition of specific antibodies in maternal colostrum and greater initial BVDV and BHV-1 serum antibody titers in their calves. We demonstrated similar results in the present study, calves from groups VACC and NO-VACC had titers of serum and nasal BRSV antibodies transferred from colostrum at 48 hours, but VACC calves demonstrated significantly greater levels of SN and nasal BRSV-IgG1 antibody titers compared with NO-VACC calves. The differences in the initial local and systemic specific BRSV antibody titers between calves in the two groups was likely the result of vaccination of cows during gestation. A greater proportion of the total passively transferred IgG1 in VACC calves corresponded to BRSV-IgG1 product of vaccination of their dams. Consequently, it is possible that IgG1 specific to other infectious agents (i.e., bacterial pathogens) was lower in VACC calves compared with NO-VACC calves because the total IgG transfer was apparently similar between groups based on serum Brix results. Results from previous studies(272, 311) suggest that total serum IgG concentration at 24-48 hours is not an accurate predictor of the transfer of pathogen-specific passive immunity in calves.

In this study, calves from VACC and NO-VACC groups had low levels of BRSV SN antibody titers on the day of challenge, and despite a small numerical difference, clinical signs of respiratory disease after challenge were not different between groups. It is unlikely that the low titers of BRSV SN antibody titers observed on the day of challenge played a significant role on clinical protection or reduced nasal shedding in VACC calves. Previous studies(9, 312) suggest that calves with Log2 BRSV SN antibody titers of 2 to 4 at the time of challenge, usually develop significant respiratory signs and nasal shedding after BRSV challenge. While viremia and nasal shedding is reduced in calves with moderate serum neutralizing antibodies against BVDV,(295, 300) serum neutralizing antibody titers against BRSV do not reliably predict BRSV shedding or extent of clinical disease.(273, 278) Additionally, nasal BRSV shedding in study calves was determined by RT-PCR, which does not assess viability nor infectivity of BRSV. Virus isolation assays to confirm shedding would have been ideal to determine if BRSV found in nasal secretions of calves was viable and infective. Factors such as upper and lower respiratory tract innate immune responses, specific BRSV antibody titers in the lung, and cell-mediated immunity, which were not evaluated in this study, could have contributed to the reduction of BRSV shedding observed in VACC calves; however, it is possible that the absence of significant clinical disease and nasal shedding observed in calves in our study corresponded to a reduced efficacy of our BRSV challenge model. In-vitro passage of BRSV through cell cultures before challenge reduces the virulence of BRSV and in this case it could have impaired viral fitness, replication, and dissemination to the lungs.(157, 313)

The rapid decay of nasal BRSV-IgG1 and the low levels of nasal BRSV-IgA before BRSV challenge suggest that after 1 month of age, nasal BRSV IgG1 and IgA derived from

colostrum likely do not play a significant role on clinical protection of calves against acute BRSV infection. We speculate that nasal BRSV-IgG1 transferred from colostrum may play an important role in clinical protection against viral infection and response to vaccination in calves under a month of age. Unfortunately, in this study the levels of nasal BRSV-IgG1 were not evaluated at additional time points during the first month of age. The dynamics of IgG1 transferred from colostrum into the respiratory tract (upper and lower) and its role on clinical protection and response to vaccination should be a matter of future investigation. Nasal BRSV-IgA increased similarly in all calves after experimental BRSV challenge reaching peak levels at day 21 post-infection. This is consistent with post-exposure or post-vaccination studies(244, 276) in BRSV naïve calves in which nasal BRSV-IgA was not detected before 8 to 10 days post-infection or vaccination. The role of nasal BRSV-IgA on protection of calves against BRSV infection and shedding is inconsistent in the literature. While in some studies(276) the presence of BRSV-IgA in the upper respiratory tract was related to reduction of clinical disease and nasal shedding, results from other studies(273, 278) show reduction of clinical disease and shedding in the absence of a significant increase of BRSV-IgA responses.

Colostrum-derived local and systemic BRSV antibodies were initially greater in VACC calves; however, similar to NON-VACC calves, their titers decayed to non-protective levels by 3 months of age and lacked of apparent residual clinical protection from BRSV challenge. Based on the results of this study, we conclude that it is unlikely that local and systemic immunity passively transferred from colostrum can provide effective clinical protection against BRSV infection in beef calves 3-months of age or older. Therefore, vaccination of beef calves at a younger age still may be necessary to reduce morbidity and mortality caused by BRSV infection

in cow-calf herds in which this pathogen contributes to the typical presentation of pre-weaning calf pneumonia between 2 and 4 months of age. We speculate that scheduling BRSV vaccination protocols for calves with adequate levels of passive immunity should strategically promote priming and boosting of local and systemic immune memory responses at times that match the highest likelihood of BRSV exposure.

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The authors declare that there were no conflicts of interest.

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Chapter 6: Local and Systemic Antibody Responses in Beef Calves Vaccinated with a Modified-Live Virus Bovine Respiratory Syncytial Virus (BRSV) Vaccine at Birth Following BRSV Infection

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David A. Martínez, Manuel F. Chamorro, Thomas Passler, Laura Huber, Paul H. Walz, Merrilee Thoresen, Gage Raithel, Scott Silvis, Ricardo Stockler and Amelia R. Woolums

Simple Summary:

Bovine respiratory syncytial virus (BRSV) is a common cause of respiratory disease in calves. Vaccination of young calves against BRSV is a common prevention strategy; however, antibodies derived from maternal colostrum interfere with vaccine response and efficacy in young calves. The objective of this study was to determine if vaccination before colostrum absorption results in the effective induction of immune responses and clinical protection in calves. Within 6 h of birth, beef calves were assigned to 2 different treatment groups. Group Vacc (*n* = 25) was vaccinated with a modified-live virus (MLV) intranasal (IN) BRSV vaccine. Group Control (*n* = 25) remained unvaccinated. At approximately 3 months of age, calves were experimentally infected with BRSV. Immune responses and viral shedding were evaluated before and after infection. Respiratory signs before and after infection as well as viral shedding

were similar between Vacc and Control calves. Local and systemic antibody responses were similar and suggested natural BRSV exposure before experimental infection. Based on the results from this study, early vaccination does not provide advantages for the clinical protection of calves from endemic BRSV farms.

Abstract:

Maternal antibodies interfere with BRSV vaccine responses and efficacy in young calves. The objective of this study was to determine if vaccination before the complete absorption of colostral antibodies results in adequate immune priming and clinical protection of beef calves. Within 6 h of life, calves were randomly assigned to 2 different treatment groups. Group Vacc (*n* = 25) received a single dose of a modified-live virus (MLV) BRSV vaccine intranasally (IN) and group Control (*n* = 25) received 2 mL of 0.9% saline IN. At approximately 3 months of age, all calves were experimentally challenged with BRSV. Serum and nasal secretion samples were collected before and after challenge for BRSV real-time RT-PCR and antibody testing. Respiratory signs were not observed before challenge. After challenge, respiratory scores were similar between groups. On the challenge day, >40% of calves in each group were febrile. The mean serum and nasal BRSV-specific antibody titers indicated natural BRSV exposure before the experimental challenge in both groups. All calves tested positive for BRSV and had a similar duration of shedding after challenge. Based on these results, vaccination at birth does not offer advantages for immune priming or clinical protection for beef calves in BRSV-endemic cow-calf herds.

Keywords: bovine respiratory syncytial virus; IgG1; IgA; antibodies; vaccine

Introduction

The bovine respiratory disease complex (BRDC) is the most common and economically important disease of beef calves in the United States (U.S.). In young beef calves, the typical clinical presentation of BRDC is pre-weaning or nursing calf pneumonia. Losses associated with BRDC within this sector of the beef industry in the U.S. have been estimated to be as high as $55 million per year (1, 14). Vaccination of calves against BRDC pathogens is a common strategy for the prevention of clinical disease; however, vaccination efficacy is variable in different production settings (5). Bovine respiratory syncytial virus (BRSV) plays an important role in the pathogenesis of BRDC and pre-weaning calf pneumonia in beef herds (314). Vaccination of young calves with intranasal (IN) modified-live virus (MLV) BRSV vaccines between 3 and 11 days of life has become a regular practice among producers and veterinarians to reduce clinical disease associated with BRSV infection in claves (265, 273, 281); however, maternal antibodies present at the time of vaccination interfere with immune priming and compromise adequate antibody responses (9). Transfer of colostral BRSV-specific immunoglobulin G-1 (IgG1) into the upper respiratory tract of young calves not only could protect against infection but also block BRSV vaccine antigens from IN vaccination. Results from a previous study demonstrated considerable levels of nasal BRSV-IgG1 at 48 h of life of beef calves that nursed colostrum from their dams (6). Additionally, results from previous studies suggest that the duration of local antibody responses (i.e., BRSV-specific immunoglobulin-A) induced by vaccination of calves with IN MLV BRSV vaccines between 3 and 11 days of age is short-lived (265, 277). The interference by pronounced levels of colostral IgG1 in the upper respiratory tract of young calves during the first week of life could prevent adequate immune priming and result in a short duration of local respiratory antibody responses, thus causing reduced efficacy of IN MLV

BRSV vaccination at an early age. The first objective of this study was to determine if vaccination of beef calves with an IN MLV BRSV vaccine within 6 h of birth before complete absorption and transfer of colostral IgG1 results in adequate priming and duration of nasal BRSV-IgA responses. The second objective was to determine if vaccination of beef calves at birth provides clinical advantages following experimental infection with BRSV.

Materials and Methods

Experimental Design

Beef calves from a single herd born to dams vaccinated at least once with a multivalent inactivated-virus BRSV vaccine (Triangle 10®, Boehringer Ingelheim Animal Health USA Inc, Duluth, GA, USA) before calving were enrolled in this study. Using dam ID numbers, calves were randomly assigned to two different treatment groups before birth; group Vacc and group Control. After calving and within 6 h of birth, 50 bull calves from the Vacc and Control groups were randomly selected for the experimental portion of the study. Group Vacc (n = 25) received a single dose (2 mL) of an intranasal (IN) modified-live virus (MLV) BRSV vaccine (Inforce 3®, Zoetis Inc., Kalamazoo, MI, USA) following the manufacturer's recommendations. Group Control (n = 25) received 2 mL of 0.9% phosphate-buffered saline (VeltivexTM® sodium chloride injection solution 0.9%, Dechra Veterinary Products; Overland Park, KS, USA) intranasally. All calves nursed colostrum naturally from their dams, and cow-calf pairs from the Vacc and Control groups were placed in separate pastures with no fence-line contact for 60 days immediately after treatment administration. Following this period, pairs from both groups were placed together in the same pasture and creep feeders were introduced to allow calves to become

familiarized with concentrate feed until early weaning. At approximately 3 months of age, all calves were weaned abruptly and transported 320 km to the experimental viral studies unit at Auburn University College of Veterinary Medicine for the challenge portion of the study. At arrival to the unit, calves were placed in a single pasture with ad libitum access to grass, fresh hay, water, and concentrate feed. Following an acclimation period of 5 days, all calves were challenged with BRSV by intranasal nebulization as described below.

Colostrum samples from dams were collected at calving to evaluate immunoglobulin G (IgG) concentrations by colostrum Brix refractometry (MA871 Digital BRIX refractometer; Milwaukee instruments; Rocky Mount, NC, USA). At 48 h of age, all calves were weighed, identified (individual electronic tag [RFID] and regular ear tag), and castrated. Additionally, blood samples were collected from all calves to evaluate transfer of passive immunity by serum Brix refractometry. Additional blood and nasal secretion samples from calves were collected before and after BRSV challenge for antibody and virological assays. The Auburn University Institutional Animal Care approved the study and Use Committee (IACUC) PRN # 2019-3550 reviewed and approved all animal protocols.

Experimental Challenge with BRSV

On Day 0, each calf was experimentally infected with 9 mL of a lung wash inoculum expanded once in Marvin Darby bovine kidney (MDBK) cells that contained 1×10^5 $TCID_{50}$ of BRSV strain GA-1/mL. Virus inoculation was performed by individual intranasal nebulization using an electronic nebulizer (Pulmo-Aide® Compressor Nebulizer System, DeVilbiss Health

Care; Washington, NY, USA) connected to a facemask (Era® Equine Mask, BIOMEDTECH, Melbourne, VIC, Australia).

Clinical Evaluation and Sample Collection

Clinical signs of disease (i.e., cough, nasal discharge, diarrhea, etc.) and treatments in study calves were recorded by on-farm personnel blinded to treatment allocation before arrival to the experimental viral studies unit and before challenge with BRSV. Following experimental challenge with BRSV, clinical evaluation and scoring was performed by a single veterinarian blinded to treatment allocation on days 0, 4, 6, 8, 10, 14, 21, and 28 relative to BRSV challenge. Blood samples were collected at 48 h and 30 days of life, and on days 0 and 28 after challenge for BRSV serum neutralization assays. Nasal secretion samples were collected at 48 h and 30 days of life, and on days 0, 4, 21, and 28 after challenge for BRSV IgG1 and IgA determination. Additional nasal secretion samples were collected on days 0, 4, 6, 8, 10, 14, 21, and 28 after challenge to determine the presence of BRSV by real time reverse transcription polymerase chain reaction (RT-PCR). Samples from each calf were labeled to ensure that treatment allocation remained masked from personnel processing samples and performing the assays.

On sampling days, clinical signs such as depression, rectal temperature, respiratory rate, cough, and nasal discharge were evaluated and a total respiratory score was assigned to each calf as previously described (305). Clinical signs were scored on a scale from 0 to 3, where 0 was considered normal (no abnormalities noted) and 3 was the most abnormal finding. Depression was scored from 0 (bright alert responsive) to 3 (obtunded, recumbent, non-responsive), rectal

temperature was scored from 0 (37.8–38.3 °C) to 3 (>39.4 °C), respiratory rate was scored from 0 (respiratory rate < 30 rpm) to 3 (respiratory rate > 100 rpm), cough was scored from 0 (none) to 3 (repeated spontaneous cough), and nasal discharge was scored from 0 (none or serous discharge) to 3 (purulent bilateral discharge). The sum of individual scores including rectal temperature, depression, respiratory rate, nasal discharge, and cough determined the presence of mild, moderate, or severe respiratory disease (305). Briefly, mild respiratory disease was determined by a sum of individual scores between 0–5, moderate respiratory disease was determined by a sum of scores between 6–10, and severe respiratory disease when the sum of scores was >10. In addition to clinical evaluation, individual body weights were obtained at birth, on day 0 (BRSV challenge), and days 14 and 28 after BRSV challenge using a portable livestock electric scale (Livestock Platform Scale® Brecknell, Fairmont, MN, USA) that was reseted to zero prior to and after each weighing.

BRSV Neutralizing Antibodies in Serum

A virus neutralization assay for detection of BRSV neutralizing antibodies on sera was performed as previously described *(282)*. Samples were heat-inactivated in a water bath, then serial 2-fold dilutions starting from 1 : 10 to 1 : 1000 were performed in 96-microwell flat-bottom plates, followed by addition of 500 µL of 100 $TCID_{50}$ BRSV to each well; all the corresponding samples dilution 3 microwells were inoculated with virus media. The plates were incubated at 37 °C in 5% CO_2 for 1 h, then MBDK cell suspension with 7% bovine serum and an antibiotic/antimycotic solution containing streptomycin, penicillin, and amphotericin B were added. The plates were incubated under the same conditions for two weeks, and the cells were evaluated daily for evidence of cytopathic effect. Results from the test were reported as the

inverse of the lowest dilution of serum required to inhibit all cytopathic effect; for the study analysis the results were Log2 transformed.

Determination of Anti-BRSV IgG1 and IgA in Nasal Secretions

Bovine respiratory syncytial viral particles were inactivated with 2 μM binary ethyleneimine, neutralized with sodium thiosulfate, and diluted 1:800 in carbonate bicarbonate buffer (pH, 9.5). The resulting solution was used to coat microwells of 96-well polystyrene plates. After coating, the plates were incubated overnight at 4 °C and washed 3 times with PBS containing 0.05% polysorbate 20 (Tween®20, Sigma-Aldrich, St. Louis, MO, USA). After washing, 200 μL of PBSS solution containing 5% sheep serum albumin (Sheep serum albumin; Sigma-Aldrich; St. Louis, MO, USA) were added to each well for blocking, then the plates were incubated at 37 °C for 1 h followed by 3 washes.

Vials containing nasal secretion samples were thawed and vortexed. Each sample was initially diluted 1 : 1 in Pluronic F127 and then diluted 1:100 in polysorbate 20. From this dilution, serial 2-fold dilutions were prepared up to 1:200 for IgG1 and up to 1:1600 for IgA and each dilution was analyzed in triplicate (i.e., each sample dilution was added to 3 wells). If the coefficient of variation among the 3 values was >20%, the outlier value was removed, and the mean value of the 2 remaining samples was used in the calculation of the antibody titer. Samples with an optical density value that was too great to be measured accurately were tested again at a higher dilution. Samples with an optical density value that was too low to be measured accurately were tested again at a lower dilution with the lowest dilution tested being 1:25 for IgA and 1:100

for IgG1. In addition to the samples, each plate had 3 microwells containing the following: positive control, which was a nasal secretion sample from a known BRSV antibody positive calf diluted 1:100 in polysorbate 20; negative control, which was low-IgG fetal bovine serum (FBS, Sigma-Aldrich; St. Louis, MO, USA) [diluted 1:100 in polysorbate 20]; and blank, which was polysorbate 20 alone. For BRSV-specific IgA, horseradish peroxidase-conjugated rabbit anti-bovine IgA (Rabbit anti-bovine IgA, Bio-Rad, Hercules, CA, USA) diluted 1:500 in an ELISA wash buffer (PBS + 0.05% TWEEN 20) and ABTS substrate solution (2,2′-azino-bis[3-ethylbenzothiazoline-6-sulphonic acid]) (ABTS, Sigma-Aldrich, St. Louis, MO, USA) were added to each well. For BRSV IgG1, horseradish peroxidase-conjugated sheep anti-bovine IgG1 (Sheep anti-bovine IgG1 HRP; Bio-Rad, Hercules, CA, USA) diluted 1:7500 in an ELISA wash buffer (PBS + 0.05% TWEEN 20) and OPD substrate were added to each well. All plates were read by a plate reader set at a wavelength of 405 nm. Wells positive for anti-BRSV IgA and IgG1 yielded a green (IgA) or yellow (IgG1) product when the bound peroxidase-conjugated rabbit anti-bovine IgA and sheep anti-bovine IgG1 reacted with the ABTS and OPD substrates, respectively. Immunoglobulin A and IgG1 titers were reported as the inverse of last dilution that was ≥2 times the mean optical density value of the negative control.

Real-Time Reverse Transcription PCR

Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed on nasal secretion samples as previously described (306). Briefly, nasal secretion sample aliquots were subjected to RNA extraction using RNAzol® (Sigma-Aldrich, St. Louis, MO, USA) following manufacturers recommendations. Once extracted, the RNA templates were reverse

transcribed and amplified with qScript™ XLT One-Step RT-qPCR ToughMix (Quantabio®, Beverly, MA, USA) using BRSV specific primers and probes (306). Each reaction (2.5 µL) was performed in a BioRad CFX96® (Bio-Rad®, Hercules, CA, USA) and results were analyzed by BioRad CFX manager® (Bio-Rad®, Hercules, CA, USA). The detection limit of the assay was established at 10^1 BRSV RNA copies/µL.

Statistical Analysis

Data were analyzed using statistical software (RStudio (Version 1.4.1717); Boston, MA, USA). The normality of the data was assessed using the Shapiro–Wilk test and examination of the residuals. Data were analyzed using generalized mixed-effects models with animal ID as the random effect, immunoglobulin titers, virus neutralization titers, rectal temperatures, and body weights as dependent variables and vaccination status and experiment time as independent variables. Post hoc familywise comparisons were performed using Tukey–Kramer with Bonferroni correction. Kaplan–Meier curves were generated to display BRSV shedding via nasal secretions over time for Vacc versus Control calves. For non-parametric variables and proportions (i.e., Brix values, clinical scores, real time RT-PCR cycle threshold values), group comparisons were performed using the Fisher's exact test, Chi-square (categorical predictor variables, 2 groups), Kruskal–Wallis (categorical predictor variables, >2 groups), and Wilcoxon Rank-Sum test (to compare medians between 2 independent populations). For all analyses, significance was set at *p*-value <0.05.

Results

Transfer of Passive Immunity and Clinical Outcomes

The median colostrum Brix value for dams of calves in the Vacc group (29.5%) was greater compared with the median Brix value for dams of calves in the Control group (26.7%); however, this difference was not statistically significant ($p = 0.12$). Similarly, the median serum Brix value for calves in the Control group (10.7%) was greater compared with that of calves in the Vacc group (10.4%); however, this difference was not statistically significant ($p = 0.18$).

Clinical disease was not recorded during the pre-weaning period and before BRSV challenge. On the day of BRSV challenge, immediately prior to inoculation, 11 calves from the Control group and 9 calves from the Vacc group presented a fever (rectal temperatures ≥ 39.7 °C). Additionally, 2 calves from the Control group and 3 calves from the Vacc group coughed repeatedly and spontaneously. Following BRSV challenge, the mean total respiratory score increased in all calves from days 0 to 8; however, significant differences were only observed in Vacc calves at 6- and 8-days post-challenge. In contrast, the mean total respiratory score decreased in both Vacc and Control calves at 21- and 28-days post-challenge; however, statistically significant differences of mean respiratory scores between Vacc and Control calves were not detected at any time point during the study (Figure 6.1). The mean rectal temperatures in calves of both groups remained elevated during the first 14 days post-challenge; however, statistically significant differences between Vacc and Control calves were not detected at any time point during the study (Figure 6.2). On day 18 post-challenge, one Vacc group calf was found dead in the pasture with no previous clinical signs of disease. Necropsy and histopathology of this calf revealed severe fibrinonecrotizing cystitis, bilateral pyelonephritis, and diffuse bronchointerstitial

pneumonia as possible causes of death. Lung tissue from this calf was determined to be negative for BRSV and other respiratory pathogens.

There was a significant effect of time on the mean individual body weights (BW) of both, Vacc and Control calves. The mean individual BW significantly increased from birth to weaning in both groups; however, statistically significant differences between Vacc and Control calves were not detected at any time point during the study (Figure 6.3). The mean average daily gain (ADG) ± SEM from birth to Day 0 in Vacc and Control calves (1.2 ± 0.04 kg/d vs. 1.3 ± 0.03 kg/d, respectively) was not significantly different between groups (p = 0.48). The mean ADG ± SEM from Day 0 to Day 28 was greater in Vacc calves compared with Control calves (0.12 ± 0.05 kg/d vs. −0.05 ± 0.08 kg/d, respectively); however, this difference was not statistically significant (p = 0.12).

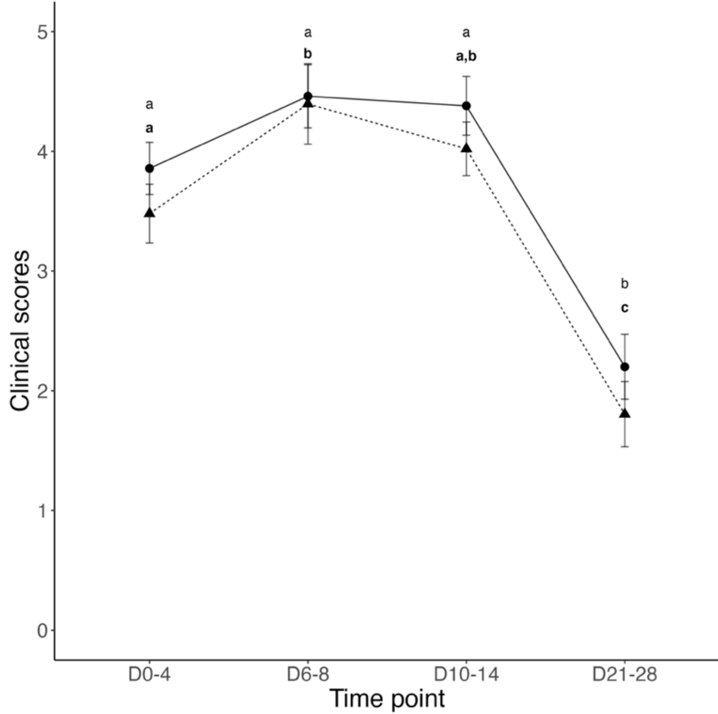


Figure 6. 1 Mean ± SEM total respiratory scores on days 0 to 4, 6 to 8, 10 to 14, and 21 to 28 after challenge of calves vaccinated intranasally (IN) with a modified-live virus (MLV) BRSV vaccine in the first 6 h of life (dashed line and triangles) versus control calves (solid line and circles) subsequently challenged with BRSV. For each group and time point, the circle or triangle represents the mean, and the whiskers represent the SEM. A line for each group connects the group's mean total respiratory score (on a scale of from 0 to 5 [none or mild respiratory disease] to >10 [severe respiratory disease]) progression throughout the study. Data were analyzed using generalized mixed-effects models. Distinct letters (small caps a, b, c) represent significant ($p < 0.05$) difference between time-points within participants of each group (Vacc in bold).

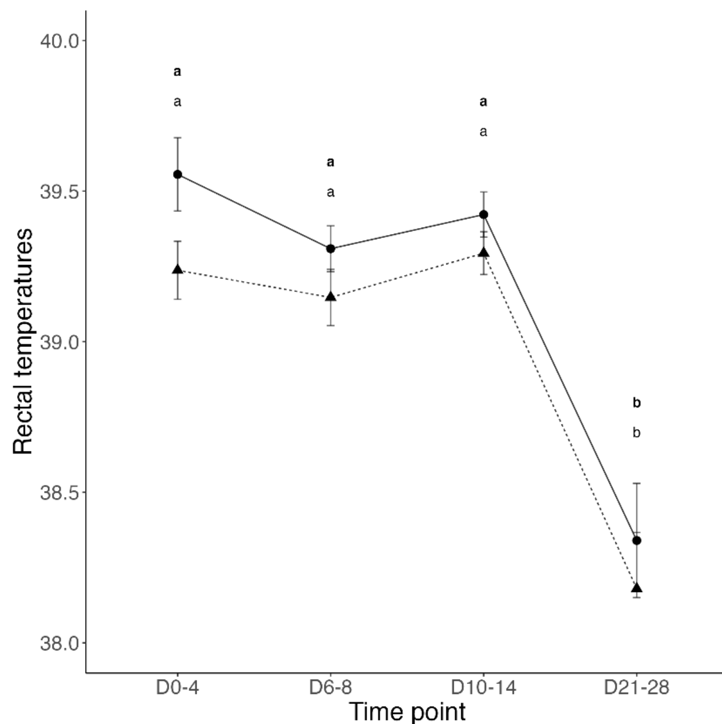


Figure 6. 2 Mean ± SEM rectal temperature (°Celsius) during days 0 to 4, 6 to 8, 10 to 14, and 21 to 28 after challenge of calves vaccinated intranasally (IN) with a modified-live virus (MLV) BRSV vaccine in the first 6 h of life (dashed line and triangles) versus control calves (solid line and circles) subsequently challenged with BRSV. For each group and time point, the circle or triangle represents the mean, and the whiskers represent the SEM. A line for each group connects the group's mean rectal temperature progression throughout the study. Data were analyzed using generalized mixed-effects models. Distinct letters (small caps a, b, c) represent significant ($p < 0.05$) difference between time-points within participants of each group (Vacc in bold).

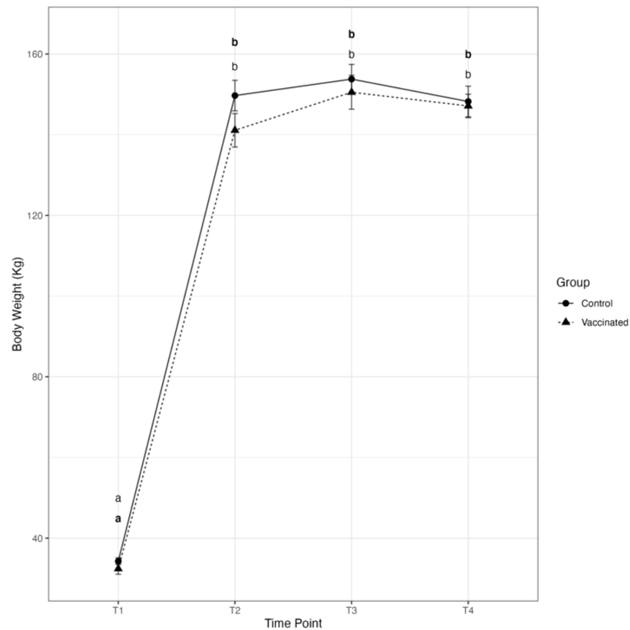


Figure 6. 3 Mean ± SEM individual body weights at T1 (birth), T2 (Day 0), T3 (Day 14), and T4 (Day 28) of calves vaccinated intranasally (IN) with a modified-live virus (MLV) BRSV vaccine in the first 6 h of life (dashed line and triangles) versus control calves (solid line and circles) subsequently challenged with BRSV. For each group and time point, the circle or triangle represents the mean, and the whiskers represent the SEM. A line for each group connects the group's mean body weight progression throughout the study. Data were analyzed using generalized mixed-effects models. Distinct letters (small caps a, b, c) represent significant ($p < 0.05$) difference between time-points within participants of each group (Vacc in bold).

BRSV Neutralizing Antibodies in Serum

The mean ± SEM Log2 BRSV serum neutralizing antibody (SNA) titers from the baseline at 48 h of life to Day 28 (last study day) were not significantly different between Vacc and Control calves at any time point. A significant BRSV SNA titer decay was observed in all calves between 48 h and 1-month of age and in the Control group between Day 0 and Day 28 (Figure 6.4-A). In contrast, a suspension of BRSV SNA decay was observed in Vacc and Control calves between 1-month of age and Day 0 (Figure 6.4-A).

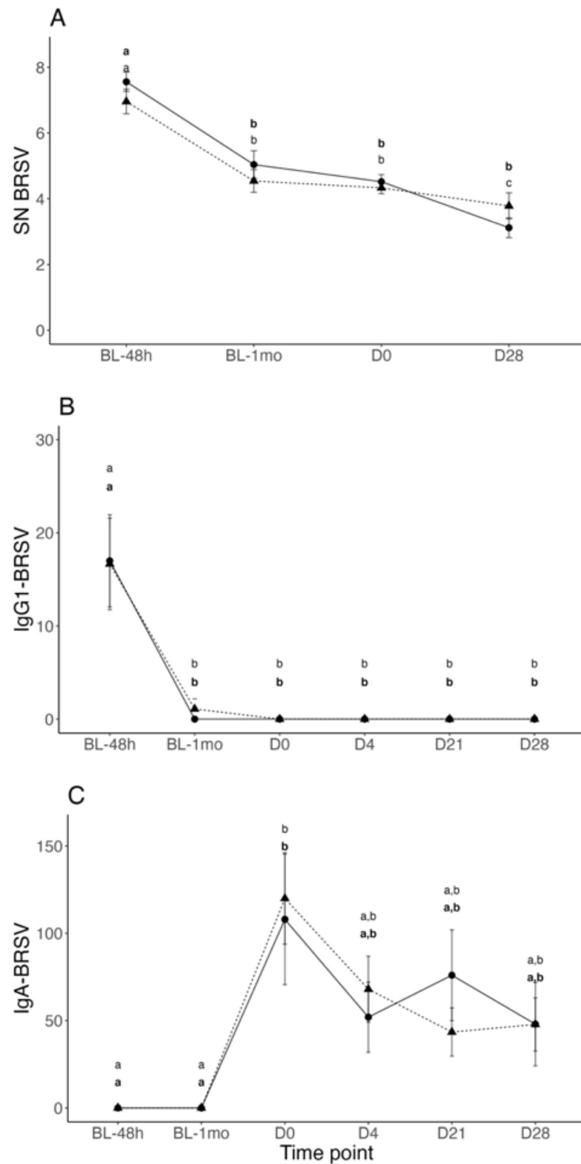


Figure 6. 4 Mean ± SEM serum neutralizing ((**A**), log_2 transformed), nasal BRSV immunoglobulin G1 (**B**), and nasal BRSV immunoglobulin A (**C**) antibody titers at 48 h of life (BL-48 h), 1-month of age (BL-1 mo), and days 0 [challenge day], 21, and 28 after BRSV challenge of calves vaccinated intranasally (IN) with a modified-live virus (MLV) BRSV vaccine in the first 6 h of life (dashed line and triangles) versus control calves (solid line and circles) subsequently challenged with BRSV. Data were analyzed using generalized mixed-effects models. Distinct letters (small caps a, b, c) represent significant ($p < 0.05$) difference between time-points within participants of each group (vaccinated in bold). Familywise multi comparisons were performed using Tukey–Kramer with Bonferroni correction. Statistically significant differences between groups were not observed at any time point.

BRSV-IgG1 and IgA Titers in Nasal Secretions

The mean ± SEM nasal BRSV IgG1 titers from the baseline at 48 h of life to Day 28 were not significantly different between Vacc and Control calves at any time point. The mean nasal BRSV IgG1 titers in both groups were high at 48 h following colostrum intake; however, after 1-month of age nasal BRSV IgG1 levels decayed to minimal levels for the reminder of the study (Figure 6.4-B). In contrast, nasal BRSV IgA titers were undetectable in Vacc and Control calves at 48 h and 1-month of age, but significantly increased in both groups on Day 0 before experimental infection with BRSV (Figure 6.4-C). Following Day 0, BRSV IgA titers significantly decayed in both groups until the end of study; however, statistically significant differences were not observed between Vacc and Control at any time point (Figure 6.4-C).

BRSV Real-Time RT-PCR in Nasal Secretions

Following BRSV challenge, nasal secretions samples from each calf in both treatment groups were confirmed to be positive to BRSV by real time RT-PCR at least once between days 4 to 10. The median and interquartile range of BRSV cycle threshold (CT) values detected by real time BRSV RT-PCR between calves from the Vacc and Control groups were not significantly different at any time point following BRSV challenge (Table 6.1). All calves were considered negative to BRSV by real time RT-PCR on days 0, 14, 21 and 28 post-challenge. The median number of days on which nasal secretions were positive for BRSV on real time RT-PCR was 2.17 days for Control calves and 2.36 days for Vacc calves, and this difference was not statistically

significant (p = 0.84). Based on real time RT-PCR results in nasal secretion samples after challenge, the risk of BRSV shedding was not significantly different (p = 0.6) between Vacc and Control calves (Figure 6.5). Ten pools of nasal secretion samples from Day 0, each representing 5 calves and both treatment groups, were submitted to the Kansas State Veterinary Diagnostic Laboratory for multiplex PCR testing including several bovine respiratory pathogens (i.e., bovine viral diarrhea virus, bovine herpesvirus 1, BRSV, bovine coronavirus, influenza D virus, *Mannheimia haemolytica*, *Mycoplasma bovis*, *Pasterella multocida*, *Histophilus somni*, and *Bibersteinia trehalosi*). Two pools, one from the Vacc group and the other from the Control group, were test-positive for *Mannheimia haemolytica* and *Pasterella multocida* with no additional positive results to any other pathogen.

Time Point	**BRSV Real Time RT-PCR CT (Median, (IQR))**		***p*-Value**
	Control	**Vacc**	
Day 0 (Challenge Day)	0	0	1.0
Day 4	32.7 (29.1, 34)	32.7 (31, 34.3)	0.85
Day 6	33.7 (32.2, 34.9)	34.5 (32.3, 36.2)	0.13
Day 8	32.9 (31.1, 34.1)	33.9 (32.1, 35)	0.36
Day 10	36.1 (33.2, 36.9)	34.9 (33.8, 36.8)	0.09
Day 14	0	0	1.0
Day 21	0	0	1.0
Day 28	0	0	1.0

Table 6. 1Median and interquartile range (IQR) of cycle threshold (CT) values for BRSV in nasal secretion samples detected by real time-RT-PCR assay for Vacc versus Control calves as described in Figure 1 from challenge day (Day 0) to Day 28 of the study.

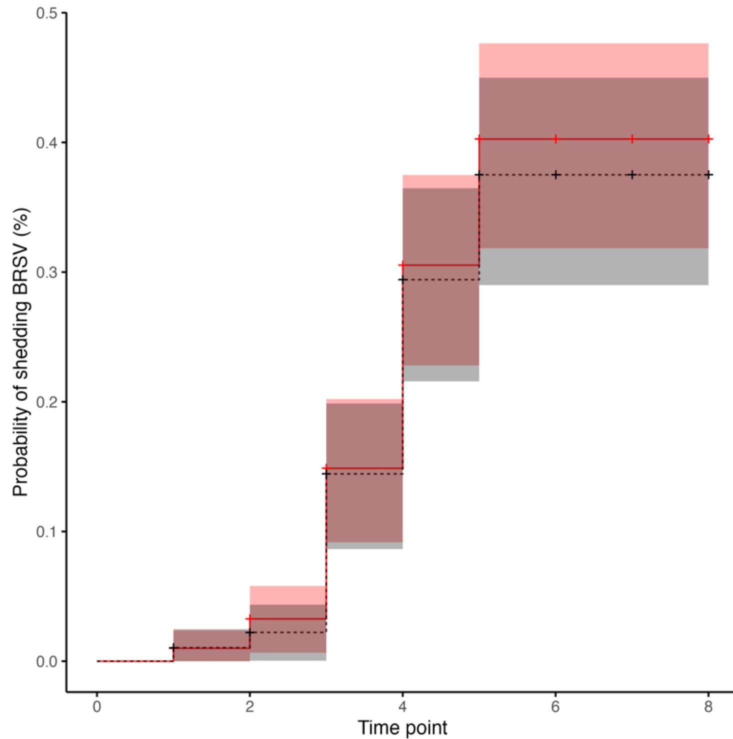


Figure 6. 5 Kaplan–Meier curves of the cumulative probability of shedding Bovine Respiratory Syncytial virus (BRSV) detected by real time-RT-PCR assay on time intervals 0 to 8, in which each time interval is representing days 0 to 2, 2 to 4, 4 to 6, 6 to 8, 8 to 10, 10 to 14, 14 to 21, and 21 to 28 after BRSV challenge of calves vaccinated intranasally (IN) with a modified-live virus (MLV) BRSV vaccine in the first 6 h of life (dashed line and triangles) versus control calves (solid line and circles) subsequently challenged with BRSV. Tick marks represent the end of each time period, each step represents detection events of BRSV shedding, and shading represents the respective 95% CI for the probability of shedding BRSV by vaccinated (red) versus control (gray) calves.

Discussion

Specific BRSV immunoglobulin A (IgA) and immunoglobulin M (IgM) responses are detected in the respiratory tract of naïve young calves between 8 and 10 days following bovine respiratory syncytial virus (BRSV) infection or intranasal (IN) modified-live virus (MLV) vaccination (244, 276). In contrast, in calves with high levels of colostrum-derived serum antibody titers, BRSV IgA in the upper respiratory tract is undetectable in the period

immediately following BRSV infection or vaccination (276); however, despite the absence of an immediate upper respiratory BRSV IgA response following vaccination, anamnestic nasal IgA responses have been reported as early as 6 days following BRSV experimental infection (276, 277). In this study, nasal BRSV IgA was not detected following vaccination, but an anamnestic nasal BRSV IgA response was detected in calves from the Vacc and Control groups on Day 0 before experimental infection with BRSV. Additionally, a suspension of the expected decay of colostrum-derived BRSV serum-neutralizing antibody titers was observed between 1 month of age and challenge day. This suggests local and systemic activation of BRSV antibody production in study calves before experimental infection with BRSV. Results from previous studies demonstrated similar dynamics for bovine viral diarrhea virus (BVDV) serum-neutralizing antibody titers when a suspension in the decay of maternal immunity was observed in 2-month-old beef calves vaccinated with an MLV BVDV vaccine in the face of maternal antibodies (IFOMA) (295, 300).

In this study, the dynamics of local and systemic BRSV antibody titers in study calves before experimental infection with BRSV suggest natural exposure to field or vaccine-origin BRSV antigens. It is possible that exposure to BRSV occurred after the commingling of calves in the same pasture 60 days after vaccination or sham vaccination and/or during transport prior to BRSV challenge. High seroprevalence rates of BRSV antibodies have been previously reported in beef cattle, and BRSV is considered endemic in some United States beef herds (201, 314). Results from one study suggested that changes in anti-BRSV serum antibody titers and seroconversion of unvaccinated cattle were the consequence of exposure to BRSV shed by subclinically infected cows or calves from the same herd (195). It is possible that the stress of commingling or transport

could have promoted BRSV shedding in subclinically infected animals in this study. Alternatively, transmission of vaccine-origin BRSV antigens from vaccinated calves to control calves following comingling could have resulted in acute infection and subsequent induction of immune responses; however, results from a previous study demonstrated nasal BRSV shedding from naïve calves vaccinated with the same vaccine used in the current study did not exceed 28 days (315). Persistence and transmission of field and/or vaccine BRSV antigens among and between cattle from commercial beef herds using IN MLV BRSV vaccines is not well understood and should be a matter of further investigation.

Clinical signs of respiratory disease (i.e., fever, cough) observed in Vacc and Control calves the day of challenge resembled a viral respiratory infection; however, attempts to identify BRSV and other viral respiratory pathogens in nasal secretion samples by real time RT-PCR on challenge day were unsuccessful. The presence of high levels of nasal BRSV IgA titers before experimental BRSV challenge strongly suggest a previous but recent natural BRSV infection before challenge. It is possible that by Day 0, the high titers of local antibody responses controlled a viral infection and eliminated viral particles from the upper respiratory tract preventing diagnosis. The presence of genetic material of *Mannheimia haemolytica* and *Pasterella multocida* in two pools of nasal secretion samples from Vacc and Control calves is difficult to interpret because these bacteria are considered normal commensals of the nasopharynx in healthy cattle (16); however, respiratory signs observed in some study calves on the day of challenge could have been the result of bacterial pneumonia associated with virulent forms of these agents. Following BRSV challenge, clinical signs of respiratory disease were mild, and shedding as well as the viral load of BRSV in nasal secretions were not different between Vacc and Control calves. It is possible

that the already elevated nasal BRSV IgA titers, present in all calves on the day of challenge, reduced viral replication, nasal shedding, and severity of clinical disease post-challenge. This is consistent with results from previous studies where a reduction in respiratory signs and nasal shedding was observed in calves with high levels of nasal BRSV IgA following experimental BRSV infection (276, 277). Additionally, other reports suggest that in cattle farms in which BRSV is endemic, repeated mucosal exposure to the virus over time increases the efficacy of local immune responses, preventing the virus from reaching the lungs and, therefore, severe respiratory disease (191, 316). The inadvertent natural exposure to BRSV before challenge prevented the evaluation of immune priming, duration of local antibody responses and clinical protection induced by vaccination at birth in this study; however, the absence of nasal BRSV IgA titers at 1-month of age in Vacc calves suggests that IN MLV BRSV vaccination as early as 6 h after birth was not sufficient to prevent maternal immunity interference. It is possible that high levels of BRSV IgG1 transferred from maternal colostrum to the upper respiratory tract prevented priming and induction of detectable levels of BRSV IgA in Vacc calves following vaccination at birth. Results from a previous study demonstrated high levels of colostrum-derived nasal BRSV IgG1 in calves that nursed colostrum from dams vaccinated with an inactivated-BRSV vaccine before calving (6); however, nasal BRSV IgG1 completely decayed before 1-month of age. This is consistent with the initial nasal BRSV IgG1 titers and decay detected in Vacc and Control calves from this study and reflects the adequate transfer of passive immunity as demonstrated by serum Brix values (307, 308). Additionally, these results highlight the relatively short half-life of specific colostral antibodies (IgG1) re-transferred to the upper respiratory mucosa in calves and their potential effect on IN MLV vaccination efficacy during this period.

Conclusions

Based on the results of this study, vaccination of beef calves with an IN MLV BRSV vaccine as early as 6 h after birth was not effective in overriding the interference from colostrum-derived antibodies and enhancing detectable BRSV-specific local antibody responses (i.e., nasal IgA) at 1-month of age. Additionally, natural exposure to BRSV may become equally effective as IN MLV vaccination at priming local and systemic immune responses in young calves from endemic beef cow-calf herds and preventing preweaning calf pneumonia as a consequence of BRSV infection. In cow-calf herds in which BRSV is not endemic vaccination of young calves may be necessary to prevent BRSV infection and disease; however, based on our results, we speculate that vaccination of young beef calves with MLV BRSV vaccines should be scheduled after 1 month of life to reduce maternal immunity interference and induce protective and possible long-lasting local and systemic immune responses.

Chapter 7: Effect of primary or booster intranasal (IN) modified-live-virus (MLV) vaccination of beef steers at 6 months of age on antibody responses, clinical protection, and detection of respiratory pathogens in nasal secretions following simultaneous challenge with bovine respiratory syncytial (BRSV) virus and bovine herpesvirus 1 (BHV-1)

Abstract

Objective

To determine the efficacy of primary or booster IN MLV vaccination at 6 months of age on antibody responses, clinical protection, and detection of respiratory pathogens in nasal secretions from single-sourced weaned beef steers following simultaneous challenge with BRSV and BHV-.

Procedures

Thirty single-sourced beef steers were randomly allocated to three different treatment groups starting at 2 months of age. Group A (n=10) was vaccinated with a single dose of a parenteral MLV vaccine and groups B (n=10) and C (n=10) remained unvaccinated. At 6-months of age, all steers were weaned and transported. Following transport, groups A and B received a single dose of an IN MLV while group C remained unvaccinated. Two days following IN vaccination, all steers where simultaneously challenged with BRSV and BHV-1. Clinical scores were recorded. Serum and nasal secretion samples were collected before and after challenge for detection of serum neutralizing antibodies and respiratory pathogens, respectively.

Results

Respiratory scores, mean Log2 serum antibody titers, and viral and bacterial respiratory pathogens in nasal secretions were not significantly different between treatment groups prior to

and following experimental challenge with BRSV and BHV-1. All calves remained negative on *Histophilus somni* and *Mycoplasma bovis* q-PCR in nasal secretion samples before IN vaccination and following vaccination and experimental challenge. While *Mannheimia haemolytica* and *Pasteurella multocida* were only detected in a moderate number of calves before IN vaccination and challenge, on day 7 days post-challenge, significantly greater bacterial loads were detected in nasal samples from all calves.

Clinical Relevance

Primary vaccination or booster with an IN MLV vaccine of beef steers at 6 months of age did not provide clinical advantages following simultaneous experimental challenge with BRSV and BHV-1. Regardless of vaccination status, weaning, transport, and BHV-1 and BRSV infection promoted an increased detection rate of *Mannheimia haemolytica* and *Pasteurella multocida* in nasal secretion samples. In contrast to previous reports, *Histophilus somni* and Myc*oplasma bovis* were not detected in this study.

Introduction:

The bovine respiratory disease complex (BRDC) continues to cause major economic losses for the beef cattle industry in the U.S.,(1, 2) and is recognized as the leading cause of death of beef calves 3 weeks of age or older.(3, 166) Bovine respiratory syncytial virus (BRSV) is one of the most prevalent respiratory viruses in U.S. cattle and plays an important role in the pathogenesis of BRDC.(4) Co-infections and synergism between viral and bacterial respiratory agents are characteristic of BRDC and, in many cases, determine disease severity and clinical outcome. Results from a previous study determined that a co-infection of BRSV and bovine viral diarrhea virus (BVDV) in calves exacerbated immune suppression, reduced oxygen exchange,

and increased the risk of secondary bacterial infection in calves.(250) In another study, BRSV-associated priming of specific T helper 2 (Th2) responses with greater levels of IgE in bronchoalveolar fluid, resulted in more severe clinical disease and greater lung pathology in calves experimentally infected with BRSV prior to challenge with *Histophilus somni* compared with calves challenged with BRSV or *H. somni* alone.(155) Additionally, results from a recent study demonstrated greater detection rates of *H. somni* in nasal secretion samples of high risk beef calves vaccinated with an IN MLV BRSV vaccine during the first 28 days following feedlot arrival compared with parenterally MLV BRSV-vaccinated and control calves.(156) Based on results from these studies, it is possible that natural BRSV infection or IN MLV BRSV vaccination could create conditions that favor *H. somni* colonization and pathogenesis; however, it is unknown if IN MLV vaccination in addition to viral infection cause additional disturbances on the commensal microbiome of the upper respiratory tract or favor the detection of other bacterial respiratory pathogens.

The majority of weaned beef calves from southeastern US cow-calf production systems are marketed through auction barns and commingled with other cattle multiple times before reaching their next stage in production.(167, 175) Commingling results in multiple opportunities for single or simultaneous exposure to respiratory viruses. Bovine herpes virus 1 (BHV-1) is another important virus of the BRDC that promotes secondary bacterial pneumonia in affected calves.(81, 88, 317, 318) Additionally, BHV-1 is present in all commercially available IN MLV vaccines labeled in the U.S. for the prevention of BRDC. Fewer than 40% of U.S. cow-calf producers vaccinate calves against respiratory viruses before weaning;[(167)] in contrast, > 99% of stocker and feedlot producers vaccinate newly acquired calves against respiratory viruses at arrival.(13, 175) Intranasal MLV respiratory vaccines induce specific mucosal immunity (IgA)

that provides local protection against infection with common respiratory viruses in cattle.(319) Most studies involving IN MLV vaccination efficacy have been performed in very young calves, and results from different clinical trials demonstrated only inconsistent protection against natural or experimental infection.(5, 273, 278, 320, 321) Factors such as interference of maternally derived immunity (IgG1) in the upper respiratory tract and short duration of mucosal immunity induced by intranasal vaccination could explain the inconsistency on clinical outcomes from IN vaccination trials.(277, 319, 322) However, intranasal MLV vaccination of southeastern beef calves around weaning time could induce short-lived but effective immunity and protection against simultaneous exposure to different respiratory viruses during their journey from the farm of origin to their next stage in production. Results from a recent study demonstrated that a booster with an IN or parenteral MLV vaccination around weaning, reduced clinical disease in calves challenged with BHV-1 4 days following to the booster.(321)

The first objective of this study was to determine if IN MLV vaccination at weaning, either used as an initial vaccination or as a booster of the primary parenteral vaccination, resulted in differences in immune responses and provided clinical advantages compared with no vaccination against simultaneous experimental challenge with BRSV and BHV-1. The second objective was to compare the detection rate of bacterial respiratory pathogens in nasal secretion samples before vaccination and following simultaneous experimental challenge with BRSV and BHV-1.

Materials and Methods

Experimental design

The Auburn University Institutional Animal Care and Use Committee (PRN No. 2021-3970) reviewed and approved all animal protocols in this study. A total of 30 crossbreed beef steers from a single farm (Upper Coastal Plains Research Unit, Auburn University, Winfield, AL) were randomly assigned to 1 of 3 treatment groups at approximately 2 months of age. Group A (n=10) received a single subcutaneous (SC) dose (2 mL) of a modified-live virus (MLV) vaccine (BOVILIS® VISTA®5 SQ; Merck & Co., Inc.) containing bovine herpesvirus 1 (BHV-1), bovine virus diarrhea virus (BVDV) types 1 and 2, bovine respiratory syncytial virus (BRSV) and parainfluenza 3 virus (PI3). Groups B (n=10) and C (n=10) received 2 mL of 0.9% SC saline (Veltivex; Dechra Veterinary Products). Group A was maintained separately from groups B and C in a different pasture until weaning to prevent inadvertent vaccine-virus transmission. At weaning (approximately 6 months of age), all calves were transported for 200 miles over approximately 6 hours to the North Auburn BVDV research Unit (Auburn University, Auburn, AL). One day after arrival to the unit (day -2), groups A and B received a single dose (2 mL) of an intranasal (IN) MLV vaccine (Nasalgen®3; Merck & Co., Inc.) containing bovine herpesvirus 1 (BHV-1), bovine respiratory syncytial virus (BRSV) and parainfluenza 3 virus (PI3). Group C received 2 mL of IN saline (Veltivex; Dechra Veterinary Products) and remained as the unvaccinated control group. Following IN vaccination, groups A and B were separated from group C in a different pasture to prevent inadvertent transmission of vaccine-viruses before challenge. Two days following IN vaccination (day 0), 15 calves (group A=5, group B=5, group C=5) were challenged with BRSV and the other 15 (group A=5, group B=5, group C=5) were challenged with BHV-1. On the following day (day 1), calves were intranasally inoculated with the opposite virus with respect to day 0. Clinical scores were recorded for all calves as described

below. Serum samples were collected before vaccination and following experimental challenge with BRSV and BHV-1 for serum neutralization analysis. Nasal secretion samples were collected before vaccination and after challenge for detection of BRSV, BHV-1, *Histophilus somni* (*Hs*), *Mannheimia haemolytica* (*Mh*), *Pasteurella multocida* (*Pm*) and *Mycoplasma bovis* (Mb).

BRSV and BHV-1 challenge

For BHV-1 inoculation, calves were placed in a squeeze chute and administered 2 mL of an inoculum containing $3x10^7$ $CCID_{50}$ of BHV-1/mL (Colorado strain) in MEM intranasally using a nasal atomizer (MAD300 Nasal Intranasal Mucosal Atomization Device, Medline Industries, LP) attached to a 3ml syringe. For the BRSV challenge, calves were placed in a hermetically sealed stock trailer measuring approximately 7.6 x 2.4 x 2.4 m with approximately 29 m^3 of air space. A lung wash inoculum of BRSV strain GA-1 with at least one cell culture passage and containing 1 x 10^5 $CCID_{50}$ BRSV/mL was delivered to all calves via 2 ultrasonic nebulizers (SU99 Elite High-Flow Ultrasonic Induction Device/Nebulizer, 115V; WestPrime Healthcare, WestPrime Inc.) placed on the opposite sides of the trailer at approximately 1.8 m above the trailer’s floor. Each ultrasonic nebulizer was loaded with 52.5 mL (~ 7 mL per calf) of inoculum that was nebulized for approximately 45 minutes. After nebulization was finished, all calves were removed from the trailer and placed in a single pasture for the rest of the study.

Clinical scoring and sampling

All calves were observed daily and clinical signs of disease (e.g., nasal discharge, cough, depression, etc.) were recorded. Clinical evaluation and scoring was performed by a single veterinarian blinded to treatment allocation on days -2, 0, 5, 7, 10, 14, 21, and 28. Clinical signs including depression, rectal temperature, respiratory rate, cough, nasal discharge and nasal erosions were evaluated on sampling days and a total respiratory score was assigned to each calf using a modification of a previously described respiratory scoring system.(305) Clinical scores were assigned in a scale of 0 to 3, where 0 was considered absent of abnormalities and 3 was the most abnormal clinical finding. Briefly depression was scored from 0 (bright alert responsive) to 3 (obtunded, recumbent, non-responsive), rectal temperature was scored from 0 (37.8-38.3°C) to 3 (> 39.4°C), respiratory rate was scored from 0 (respiratory rate < 30 rpm) to 3 (respiratory rate > 100 rpm), cough was scored from 0 (none) to 3 (repeated spontaneous cough), nasal discharge was scored from 0 (none or serous discharge) to 3 (purulent bilateral discharge) and nasal erosions were scored from 0 (none or absent) to 3 (bilateral plaques [at least 2 or more per nostril or median septum] with fibrin deposition). The sum of individual scores including depression, rectal temperature, respiratory rate, cough, nasal discharge and nasal erosions determined the presence of mild, moderate, or severe respiratory disease.(305) Mild respiratory disease was determined when the sum of scores was between 0-5, moderate respiratory disease was determined when the sum of scores was between 6-10, and severe respiratory disease when the sum of scores was > 10. Individual body weights were recorded on days -2, 14, and 28 using an electronic portable livestock scale (Livestock Platform Scale® Brecknell, Fairmont, MN, USA) that was zeroed prior to weighing each animal.

Serum samples were collected at 2 months of age, and on days -2 and 28 of the study for serum neutralization assays. Nasal secretion samples were collected on days -2, 5, 7, 11, 14, and

28 to determine the presence of BRSV, BHV-1, *Hs*, *Mh*, *Pm*, and *Mb* using quantitative PCR assays for each pathogen. Samples from each calf were labeled such that treatment allocation remained blinded from personnel processing samples and performing laboratory assays.

Virus neutralization

Serum neutralization for BRSV was performed as previously described.(282) Serum samples were heat-inactivated in a water bath at 55°C for 30 minutes, then serial 2-fold dilutions from 1:2 to 1:1024 were performed in 96-microwell flat-bottom plates, and 50 μL of 200 $CCID_{50}$ of BRSV GA-1 were added to wells. For each dilution, equal volumes of virus culture media were added to 3 wells. After dilution, the plates were incubated at 37°C in 5% CO_2 for 1 hour, and MBDK cells suspended in minimum essential medium (7% bovine serum and a solution containing streptomycin, penicillin, and amphotericin B) were inoculated. The plates were re-incubated every 96 hours and monitored daily for the presence of cytopathic effect by microscopic evaluation. For BHV-1 serum neutralization, 96-microwell flat-bottom plates were inoculated with an equal volume (50 μL) of culture media containing 100-500 $CCID_{50}$ of Colorado strain of BHV-1, then each well was inoculated with the respective serum dilution and incubated for 96 hours at 38.5°C in a humidified atmosphere of 5% CO2 and air. The plates were examined microscopically for the characteristic cytopathic effect of BHV-1 every 96 hours. Antibody titers were reported as the inverse of the lowest dilution of serum required to inhibit all cytopathic effect and results were Log2 transformed for statistical analysis.

Real-time, quantitative (q), reverse transcription (RT) polymerase chain reaction (PCR) for BRSV

Real time qRT-PCR was performed in nasal secretion samples as previously described.(306) Briefly, RNA extraction from nasal secretion samples was performed using a commercially available reagent (RNAzol®, Sigma-Aldrich) following the manufacturer's recommendations. Once extracted, the RNA templates were reverse transcribed and amplified with qScript™ XLT One-Step RT-qPCR ToughMix (Quantabio®, Beverly) using BRSV specific primers and probes. Each reaction (2.5 μL) was performed in a BioRad CFX96® system (Bio-Rad®, Hercules) and results were analyzed by BioRad CFX software manager® (Bio-Rad®, Hercules). The detection limit of the assay was established at 10^1 BRSV RNA copies/μL.

Real-time qPCR for BHV-1

A membrane kit was used to extract viral DNA from nasal secretions samples according to the manufacturer's instructions. A SYBR Green-based real-time PCR protocol was developed to detect BHV-1 as previously described.(295) The optimal PCR conditions were determined to be the lowest concentration of the standard template required to amplify the correct gene fragment corresponding to the virus. Forward and reverse BHV-1 primers were used. The reaction mixture contained 5μL of DNA template, 10 μL of Sso Advanced universal SYBR Green Ssupermix, 0.5 μM (0.5 μL), forward and reverse primers for BHV-1, and sterile PCR-grade water for a total volume of 20 μL. The PCR protocol for detection of BHV-1 was 1 cycle at 98ºC for 3 min followed by 95 ºC for 15s and 60 ºC for 3s for 35 cycles. The standard DNA template served as positive control. The non-template control served as negative control. All the reactions were

performed in a real-time PCR detection system (BioRad CFX96® system, Bio-Rad®, Hercules) and results were analyzed by BioRad CFX software manager® (Bio-Rad®, Hercules). The detection limit of the assay was established at 10^1 BHV-1 DNA copies/µL.

Real time qPCR for bacteria

Total DNA was extracted from nasal secretion samples using the MagMAX™-96 Viral RNA Isolation Kit AM1836 (ThermoFisher Scientific, USA) and purified using Program AM1836-DW-One. Upon completion of the program, the plate with purified DNA was placed on ice for further testing.

Each sample was tested for *Hs*, *Mh*, *Pm*, and *Mb* by multiplex real time PCR immediately after DNA purification. Real-time PCR was carried out using the Path-ID Multiplex One Step RT-PCR kit (Life Technologies, USA) for the multiplex reactions (*Mh*, *Pm*, and *Hs*) and the Path-ID qPCR Master Mix for the *Mb* reaction. The *Mh* and *Pm* multiplex reaction contained a 25X primer-probe mix (PPM) with 22.5 µM concentration of primers, 5.0 µM concentration of probes for the bacterial targets, and 5 µM and 3.1 µM concentration of XIPC primers and probe, respectively. The second multiplex reaction contained a 25X PPM with 22.5 µM concentration of primers and 5.0 µM concentration of probe for *Hs,* and 5 µM and 3.1 µM concentration of XIPC primers and probe, respectively. The *Mb* reaction contained a 25X PPM with 11.25 µM concentration of primers and 3.13 µM concentration of probe for *Mb,* and 5 µM and 3.1 µM concentration of XIPC primers and probe, respectively. For the multiplex reactions, the PCR mix contained 1µl of nuclease-free water, 12.5 µl of 2X MP RT-PCR Buffer, 1.0 µl PPM, 2.5 µl of 10X MP enzyme mix, and 8 µl of purified DNA. For the *Mb* reaction, the PCR mix contained 3.5 µl of nuclease-free water, 12.5 µl of 2X qPCR Master Mix, 1.0 µl of PPM, and 8 µl of

purified DNA. The cycling conditions for all reactions were as follows: 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 45 sec with data collection at the second amplification step. The sequences of the primers and probes used in the real time PCR reactions have been previously published for XIPC,(323) *Mb*,(324) *Mh*,(325) *Hs* (326),(327). All real time PCR reactions were carried out on a ABI7500 Fast Real-Time PCR System (ThermoFisher Scientific, Waltham, MA USA) using standard plasticware. Negative extraction control (NEC), no template control (NTC) and positive amplification control (PAC) were utilized. Samples were considered "detected" for the targeted pathogens if Ct values were below 36, there was no amplification in the NEC and NTC, the Ct value for the PAC was 24-34, and XIPC Ct value for each sample was below 40. Samples with target Ct values above 36 and XIPC Ct values below 37 were considered "not detected".

Statistical analysis

Data were analyzed using statistical software (RStudio Version 1.4.1717; Boston, MA). The normality of the data was assessed using the Shapiro-Wilk test and examination of the residuals. Generalized mixed-effects and mixed-effects logistic regression models were used to evaluate the effect of vaccination status and experiment time-point on the sum clinical scores, body weight, BRSV and BHV-1 neutralization antibodies in serum, and on the detection of BRSV, BHV-1, and bacteria in nasal secretions samples. We used animal ID as the random effect to account for repeated measurements. Post-hoc familywise comparisons were performed using Tukey-Kramer with Bonferroni correction. Kaplan–Meier curves were generated to display BRSV, BHV-1, and bacteria detection in nasal secretions over time for calves in groups A, B and C. Line graphs for categorical variables (for example, detection of BRSV, BHV, and bacteria by

PCR techniques) were constructed by calculating the proportion of positives from all samples tested. For all analyses, significance was set at p-value < 0.05.

Results

Clinical scores and body weights

Adverse reactions to vaccination or clinical signs of disease were not observed before viral challenge. Mortality was not observed during the entire study period. Following challenge with BRSV and BHV-1, signs of respiratory disease including fever (rectal temperature > 39.4°C), tachypnea, cough, nasal discharge and nasal erosions were moderate, and the mean respiratory scores were significantly increased ($P < 0.05$) in all treatment groups on days 5, 7, 10, and 14 after challenge with a peak on days 7 and 14 (Figure 7.1). Between days 7 and 14 post-challenge, the mean respiratory score for group A was lower compared with that of groups B and C; however, statistically significant differences between groups were not detected at any time point.

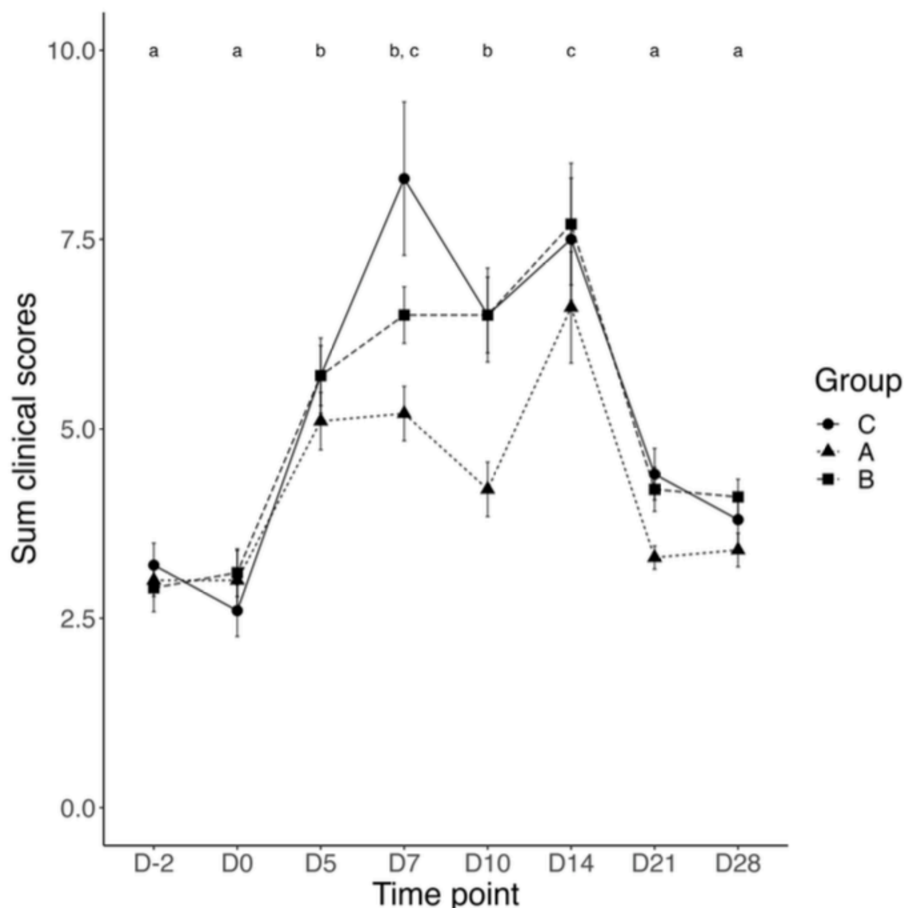


Figure 7. 1 Mean ± SEM total respiratory scores on days -2 (D-2), 0 (D0), 5 (D5), 7 (D7) 10 (D10), 14 (D14), 21 (D21) and 28 (D28) before and after experimental infection with BRSV and BHV-1 on day 0 for calves in the control (C) and vaccinated groups (A and B). For each group and time point, the circle, triangle, or square represents the mean, and the whiskers represent the SEM. A line for each group connects the group's mean clinical score progression throughout the study. Data were analyzed using generalized mixed-effects models. Distinct letters represent significant ($P < 0.05$) difference between time-points within participants of each group.

The mean ± SEM individual body weights (BW) in kg increased in all treatment groups overtime throughout the study period; however, a statistically significant increase in BW was detected from day -2 to day 14 ($P < 0.05$), but not from day 14 to day 28 (Figure 7.2). Statistically significant differences of mean BW between treatment groups were not detected at any time point. The mean average daily gain (ADG) ± SEM from day -2 to day 28 (A = 1.59 ±

0.4 kg/d vs. B = 1.67 ± 0.3 kg/d vs. C = 1.92 ± 0.3 kg/d) was not significantly different between treatment groups (P = 0.08).

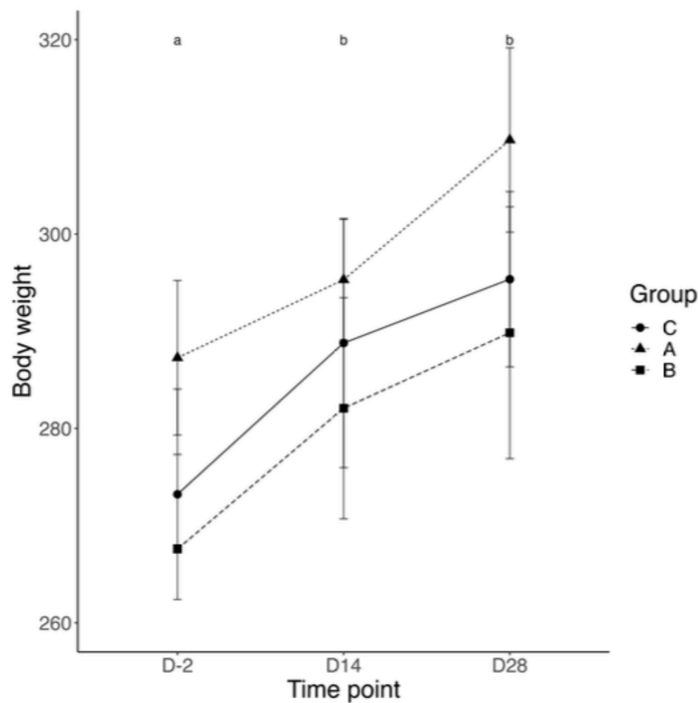
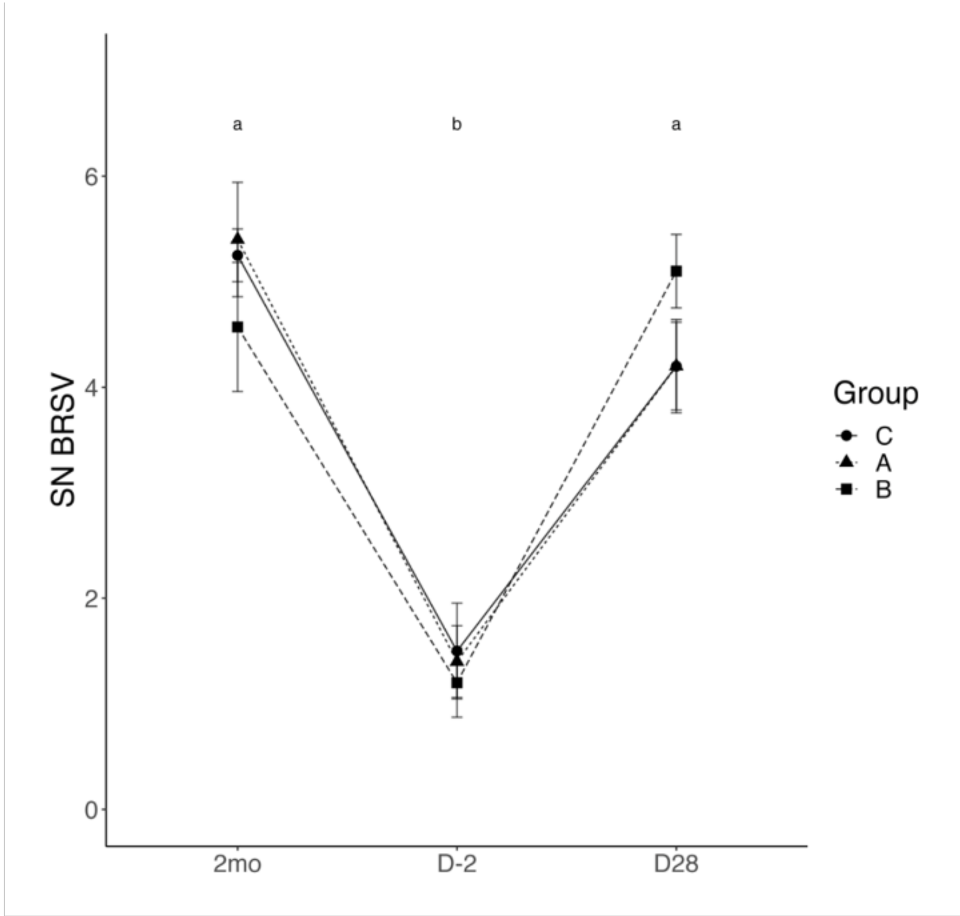


Figure 7. 2 Mean ± SEM body weights on day -2 (D-2), and on days 14 (D14) and 28 (D28) for calves in the control (C) and vaccinated groups (A and B). For each group and time point, the circle, triangle, or square represents the mean, and the whiskers represent the SEM. A line for each group connects the group's mean clinical score progression throughout the study. Data were analyzed using generalized mixed-effects models. Distinct letters represent significant ($P < 0.05$) difference between time-points within participants of each group.

BRSV and BHV-1 neutralizing antibodies in serum

Moderate mean ± SEM Log2 BRSV serum neutralizing antibody (SNA) titers were present in all treatment groups at 2 months of age (A = 5.4 ± 0.5 vs. B = 4.3 ± 0.4 vs. C = 5 ± 0.5); in contrast, low mean ± SEM Log2 BHV-1 SNA titers were present in all treatment groups

at the same time point (A = 1.7 ± 0.6 vs. B = 1.4 ± 0.6 vs. C = 0.5 ± 0.3). A statistically significant effect of time ($P < 0.05$) was detected in the mean Log2 BRSV SNA titers between 2 months of age and day -2 and between day -2 and day 28. In all treatment groups, the mean Log2 BRSV SNA decayed by day -2 compared with titers present at baseline at 2 months of age; however, BRSV SNA titers increased following vaccination and viral challenge on day 28 (Figure 3A). A similar statistically significant effect of time was detected in the mean Log2 BHV-1 SNA titers between day-2 and day 28. In all treatment groups, the mean Log2 BHV-1 SNA titers following viral challenge increased by day 28 compared with the previous time points (Figure 7.3B). Statistically significant differences in mean Log2 BRSV and BHV-1 SNA among treatment groups were not observed at any time point during the experiment (Figures 7.3A and B).


a
b
a
SN BRSV
6
4
2
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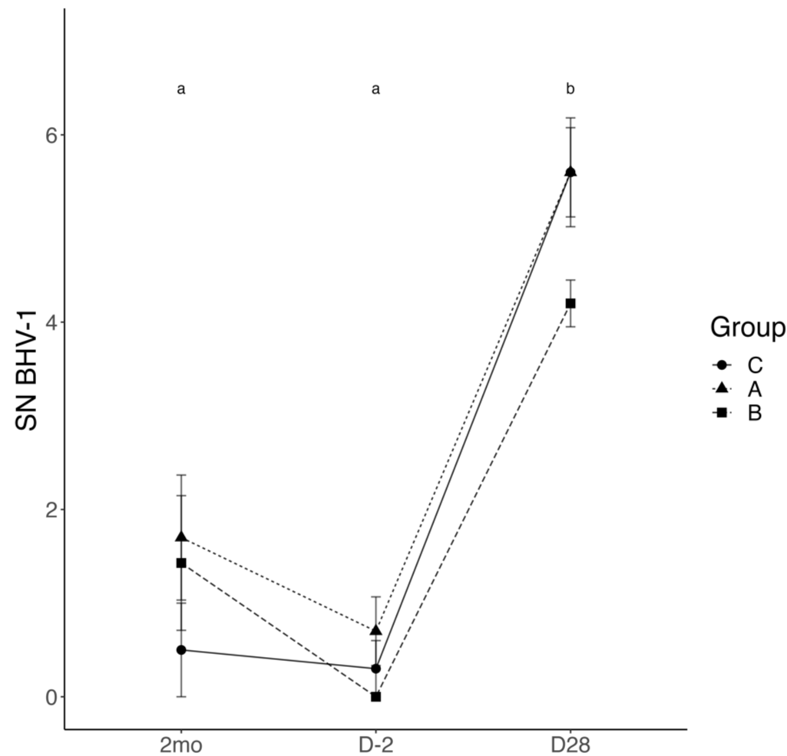


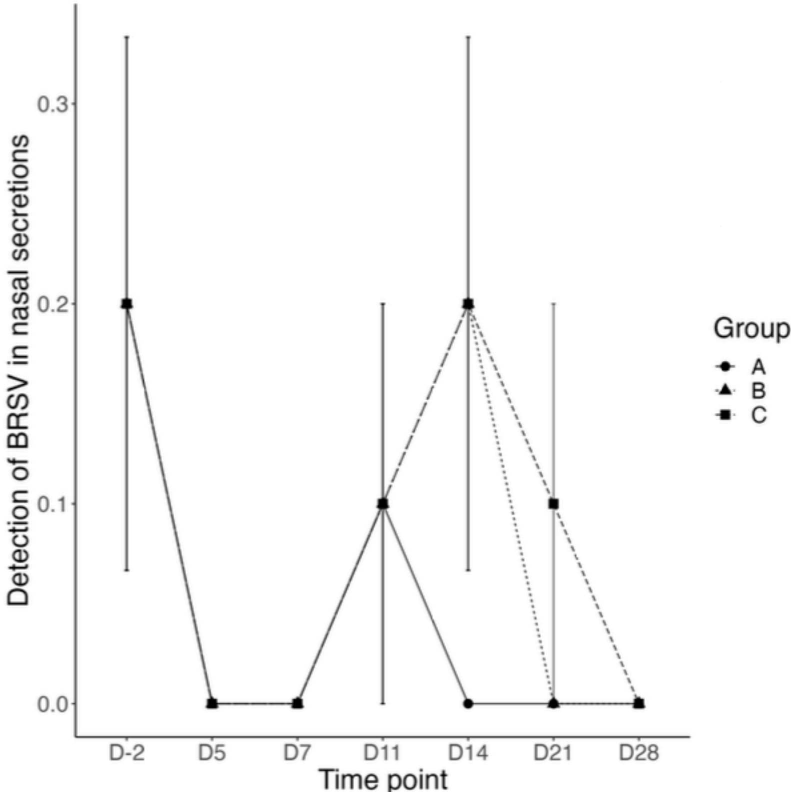
Figure 7. 3 Mean ± SEM BRSV (panel A) and BHV-1 (panel B) SNA titers for calves in the control (C) and vaccinated groups (A and B) at 2 months of age (2mo), 2 days prior viral challenge (D-2) and day 28 (D28) of the experiment. Data were analyzed using generalized mixed- effects models. Distinct letters represent significant ($P < 0.05$) difference between time-points within participants of each group. Familywise multi comparisons were performed using Tukey-Kramer with Bonferroni correction. Statistically significant differences between groups were not observed at any time point.

Detection of genetic material of BRSV and BHV-1 in nasal secretions by PCR assays

On Day -2, 20% of calves in each treatment group were positive by BRSV RT-PCR in nasal secretion samples. At the same time point, 80%, 70%, and 70% of calves from groups A, B and C, respectively were positive by BHV-1 PCR in nasal secretion samples. At every sampling point starting at day -2, a lower proportion of calves were positive to BRSV in nasal secretions samples compared with the proportion of calves positive to BHV-1 across all treatment groups

(Figures 4A and B). The proportion of calves positive for BRSV in nasal secretions was low and did not significantly vary from days -2 to 28 (Figure 7.4A). In contrast, a significantly lower ($P < 0.05$) proportion of calves were positive for BHV-1 on days 21 and 28 of the study compared with days -2, 5, 7 and 11 (Figure 7.4B). Statistically significant differences among treatment groups were not detected for any of the viruses at any time point.

Based on detection of viruses by PCR assays in nasal secretion samples from calves in all treatment groups between days -2 and 28, the risk of shedding BRSV and BHV-1 was not significantly different ($P = 0.6$ and 1, respectively; Figures 7.5A and B) across time, and statistically significant differences were not detected among treatment groups at any time point.



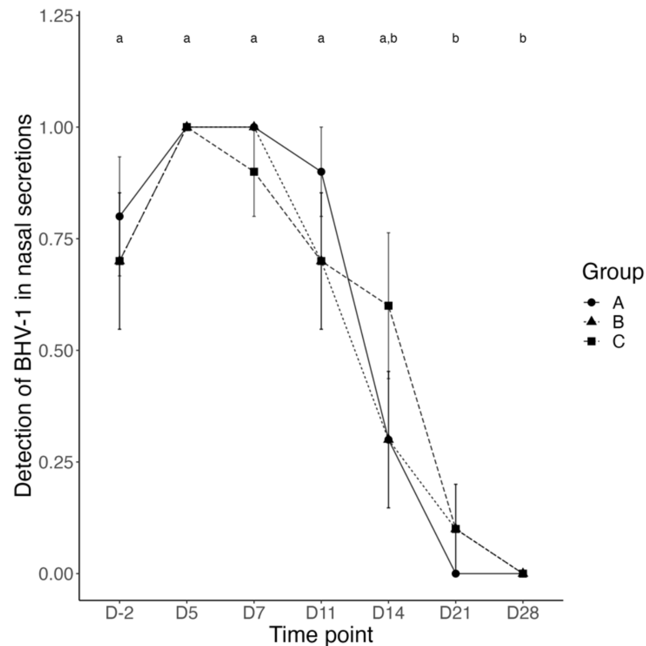
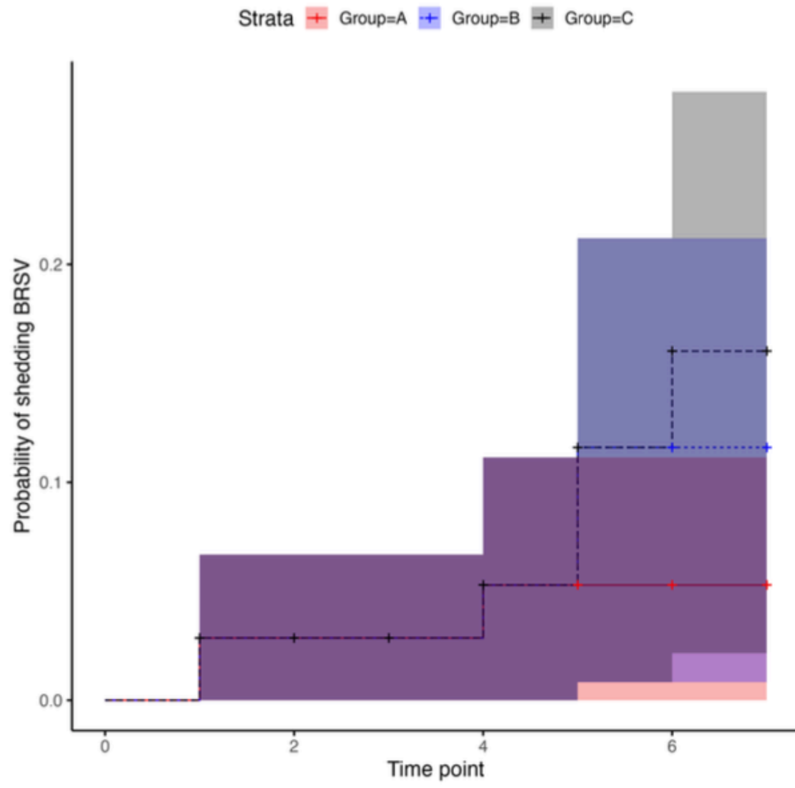


Figure 7. 4 Mean proportion ± SEM of calves positive to BRSV (panel A) and BHV-1 (panel B) in control (C) and vaccinated groups (A and B) 2 days before viral challenge (D-2) and on days 5 (D5), 7 (D7), 11 (D11), 14 (D14), 21(D21) and 28 (D28) following challenge. Data were analyzed using mixed-effects logistic regression. Distinct letters represent significant ($P < 0.05$) differences between time-points within participants of each group. Familywise multi comparisons were performed using Tukey-Kramer with Bonferroni correction. Statistically significant differences between groups were not observed at any time point.


Strata
Group=A
Group=B
Group=C
Probability of shedding BRSV
0.0
0.1
0.2
0
2
4
6
Time point

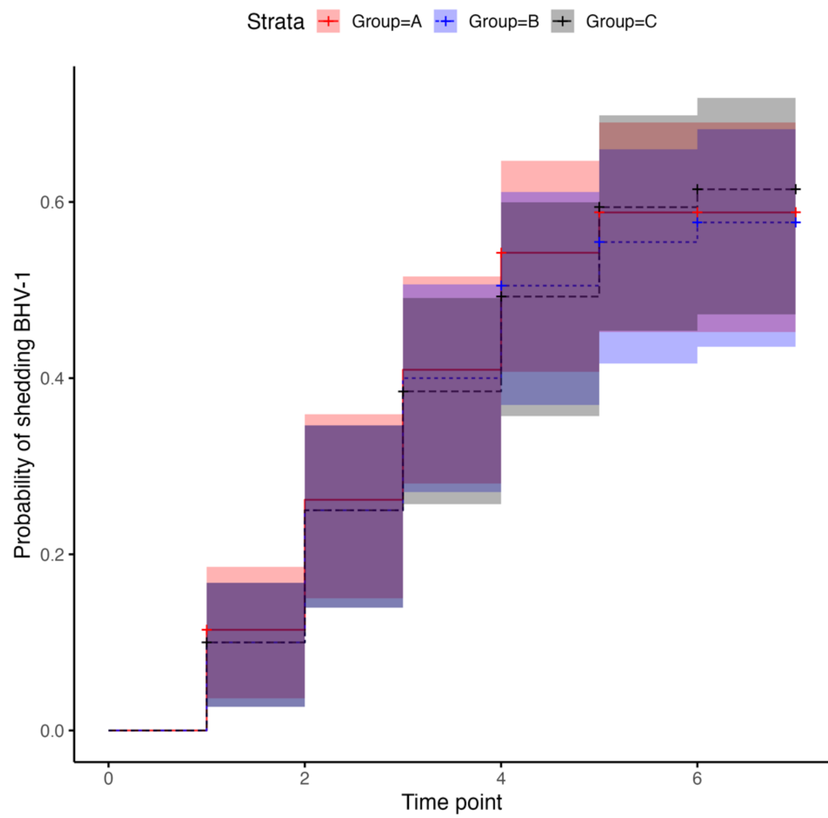
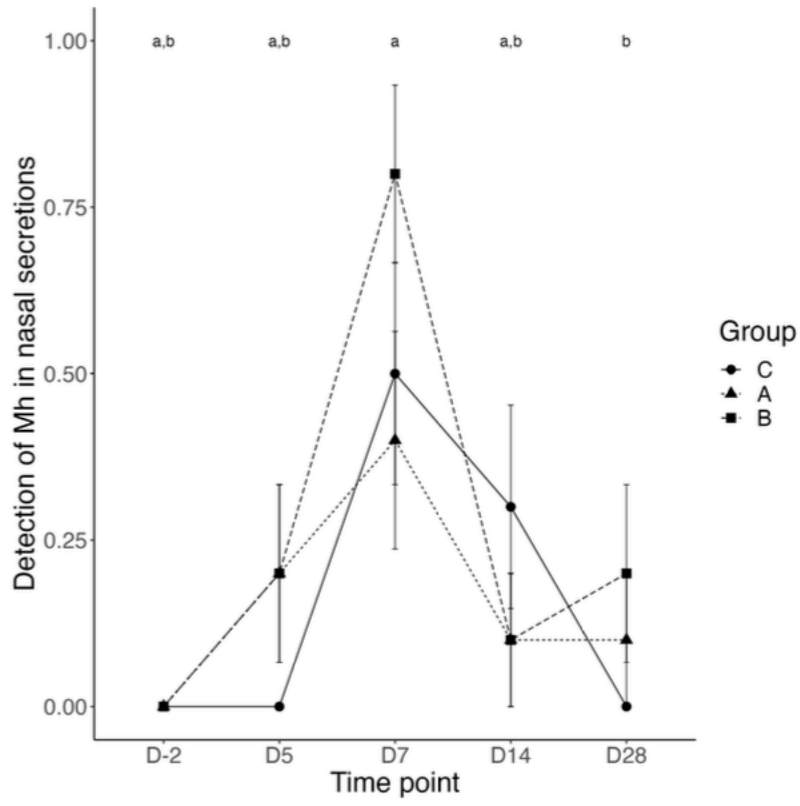

Strata
Group=A
Group=B
Group=C
Probability of shedding BHV-1
0.0
0.2
0.4
0.6
0
2
4
6
Time point

Figure 7. 5 Kaplan-Meier curves of the cumulative probability of shedding BRSV (A) and BHV-1 (B) detected with RT-PCR and PCR, respectively for calves in control [(C) gray] and vaccinated groups [A (red) and B (purple)] on time intervals 0 to 7, in which each time interval is representing days -2 to 0, 0 to 5, 5 to 7, 7 to 11, 11 to 14, 14 to 21 and 21to 28 following viral challenge. In each graphic, tick marks represent the end of each time period, each step represents detection events of BRSV and BHV-1 shedding, and shading represents the respective 95% CI for the probability of shedding BRSV and BHV-1 by calves.

Detection of genetic material of *Histophilus somni, Mannheimia haemolytica, Pasteurella multocida* and *Mycoplasma bovis* in nasal secretions by PCR

Histophilus somni and *Mb* were not detected in nasal secretion samples from any calf in any treatment group from day -2 (before IN vaccination) until day 28 following viral challenge. On day -2, *Mh* was not detected in nasal secretion samples from calves in group A, and *Mh* was minimally detected (Ct range $> 35 \leq 45$) in 11% of calves from groups B and C. In contrast, on day -2 *Pm* was detected in 100% of calves of groups A, B, and C. Following day -2, the rate of detection of *Mh* and *Pm* in nasal secretion samples from calves in all groups increased until day 7 after viral challenge (Figures 7.6A and B). The odds of *Mh* detection in nasal secretion samples were significantly greater ($P < 0.05$) on day 7 compared with day 28 after viral challenge across all treatment groups (Figure 7.6A). Similarly, the odds of *Pm* detection in nasal secretion samples were significantly greater ($P < 0.05$) on day 7 compared with days 14 and 28 following viral challenge (Figure 7.6B). Statistically significant differences between treatment groups were not observed at any time point.


a,b
a,b
a
a,b
b
1.00
0.75
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Detection of Mh in nasal secretions
D-2
D5
D7
D14
D28
Time point
Group
C
A
B

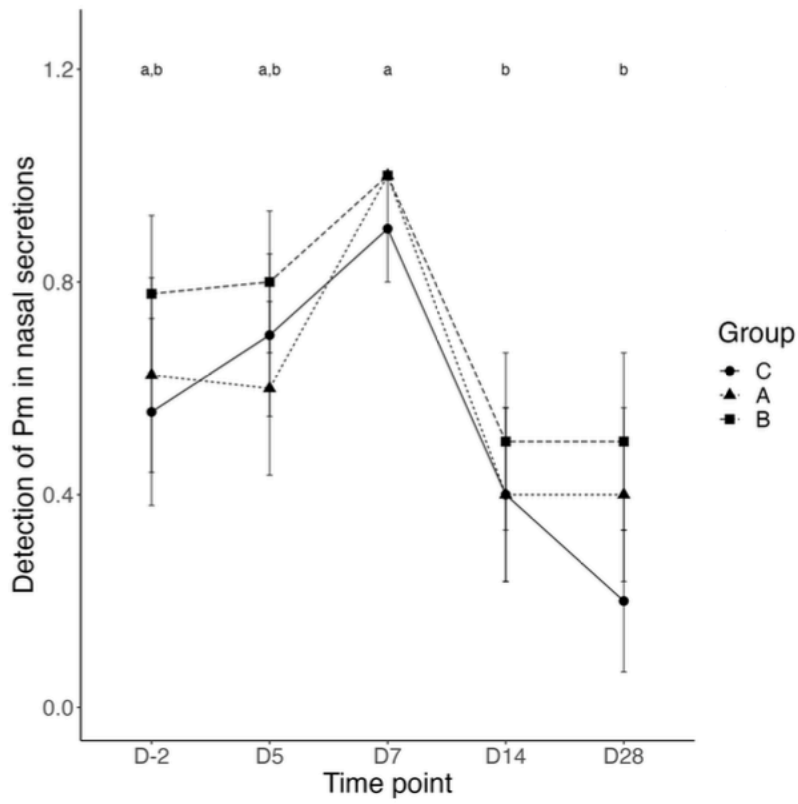
a,b
a,b
a
b
b
1.2
0.8
0.4
0.0
Detection of Pm in nasal secretions
D-2
D5
D7
D14
D28
Time point
Group
C
A
B

Figure 7. 6 Mean proportion ± SEM for *Mannheimia haemolytica* (*Mh*) (panel A) and *Pasteurella multocida* (*Pm*) (panel B) detection for calves in control (C) and vaccinated groups (A and B) on days -2, 5, 7, 14, and 28 of the experiment. Data were analyzed using mixed-effects logistic regression. Distinct letters represent significant ($P < 0.05$) difference between time-points within participants of each group. Familywise multi comparisons were performed using Tukey-Kramer with Bonferroni correction. Statistically significant differences between treatment groups were not observed at any time point.

Discussion

In this study, our goal was to replicate some of the conditions typical of cow-calf and stocker beef production systems of the southeastern United States (U.S.) that lead to a greater risk of BRDC in this group of cattle. Administering an IN MLV vaccine to high-risk beef calves of unknown health or vaccination status at arrival to stocker farms can be an alternative to prime local mucosal immunity and reduce mortality due to BRDC during the first weeks after arrival. Results from a previous study demonstrated that primary vaccination or booster (4.5 months after primary vaccination) with an IN MLV vaccine at weaning reduced mortality due to *Mannheimia haemolytica* pneumonia in beef calves challenged with BHV-1 4 days following IN MLV vaccination.(321) Additionally, a combination vaccine protocol with an IN MLV vaccine at 3-6 weeks and an IN MLV booster at 6 months resulted in significant reduction of clinical disease following challenge of calves with BHV-1 4 days post booster.(321) In our study, the mean respiratory score on day 7 post-challenge was greater in unvaccinated control calves (group C) compared with calves that received a combination vaccine protocol at 2 months and 6 months (group A) and calves only vaccinated at 6 months (group B); however, these differences were not statistically significant. It is possible that the onset of protection from IN MLV vaccination 2

days prior to challenge was not sufficiently expeditious, and that the BRSV and BHV-1 infection overwhelmed limited local innate and/or anamnestic adaptive responses potentially induced by vaccination. In another study, respiratory scores of beef calves, challenged with BRSV at 6 months of age that had been vaccinated with an IN MLV vaccine at birth and a parenteral KV or MLV vaccine at 2 months of age, were not statistically different compared with unvaccinated calves.(273) The short duration of mucosal immunity induced by IN MLV vaccination and the short time between vaccination and viral exposure could have prevented triggering adequate immune responses that provided a significant reduction of respiratory signs in vaccinated calves; however, results from two studies demonstrated that a combination vaccine protocol or primary IN vaccination shortly before viral challenge with BRSV or BHV-1 reduced calf mortality despite the lack of significant effects on clinical signs.(273, 321)

On day -2 before IN vaccination and viral challenge, 20% of calves were q-PCR positive for BRSV and more than 70% of calves were positive for BHV-1 in nasal secretion samples. It is possible that the stress of weaning and transport shortly before IN vaccination contributed to viral shedding and transmission between calves from different treatment groups in this study. Results from previous studies suggested that shedding and transmission of BRSV and BHV- 1 from chronically or latently infected cattle can occur in endemic farms where BRSV and BHV-1 circulate amongst cattle populations.(290, 322, 328) Additionally, latent infection with BHV-1 has been reported in cattle previously vaccinated with MLV BHV-1 containing vaccines, and BHV-1 shedding occurs in latently infected cattle following stressful events or immunosuppressive treatment.(328, 329) It is possible that some of the calves from group A that were vaccinated with a parenteral MLV BHV-1 at 2 months of age, developed a latent BHV-1 infection and shed the virus following weaning and transport. Primary IN MLV vaccination or

IN MLV booster at 6 months of age, 2 days prior to viral challenge, did not result in a significant reduction of BRSV and BHV-1 detection in nasal secretion samples from beef calves during the entire study period. This contrasts with results from previous studies that demonstrated reduction of BRSV and BHV-1 nasal shedding following challenge in calves vaccinated as close as 4 days prior to challenge with combination vaccine protocols at birth and 2 months, at 2 months and 6 months, or primary vaccination at 6 months.(273, 321) It is possible that IN MLV vaccination 2 days prior to challenge contributed to nasal detection of BRSV and BHV-1 in nasal secretions of groups A and B in our study. Results from a recent study demonstrated a 100% positive rate for BHV-1 and BRSV by PCR between 3 and 21 days in calves vaccinated with IN MLV vaccines.(315) Additionally, as previously discussed, it is possible that IN MLV vaccination 2 days prior to viral exposure did not allow enough time to trigger protective innate or anamnestic immune responses that resulted in significant reduction of viral replication and clinical disease. Developing protective immune responses in the upper respiratory tract against multiple viral pathogens following vaccination shortly before (weaning day) or the day of exposure/challenge (at arrival) may be exhaustive and impossible for the immune system of highly stressed 6-month-old weaned beef calves marketed through auction barns.

Compared with BRSV, a greater proportion of calves were positive by BHV-1 q-PRC in nasal secretion samples across all treatment groups following IN vaccination and simultaneous viral challenge. This could be associated with the greater antigen load of BHV-1 compared with BRSV inoculated to calves within our challenge model; however, BHV-1 has a strong tropism for the upper respiratory tract of cattle and its ability to replicate and move promptly transcellularly across epithelial cells could have negatively affected BRSV replication and detection in nasal secretions. Additionally, based on results from previous studies, the probability

of detecting BRSV by RT-PCR in nasal secretion samples of calves with BRDC is lower compared with transtracheal wash or bronchoalveolar fluid samples.(259, 260) Unfortunately, we did not collect lower respiratory tract fluid samples to compare the rate of detection of both viruses in each anatomical location. Based on the results of this study it is possible that BHV-1 overtook replication in the upper respiratory tract of challenged calves compared with BRSV. The responses to IN MLV may be impacted if BHV-1 overtakes replication in the upper respiratory tract compared with other viruses (i.e., BRSV and PI3), thus skewing clinical protection towards one virus.

Regardless of vaccination status, serum neutralizing antibody titers (SNA) to BRSV and BHV-1 decayed from 2 months to 6 months of age (day -2) across all treatment groups and without significant differences at any time point. This reflects interference on the induction of antibody responses to parenteral MLV vaccination in group A by the presence of moderate and minimal levels of BRSV and BHV-1 SNA, respectively, at 2 months of age. The presence of colostrum-derived antibodies in serum at the time of parenteral or IN MLV vaccination in calves may inhibits local and systemic humoral immune responses.(9, 276, 295, 322) Previous studies demonstrated similar SNA titer results for BRSV and BHV-1 in 6-month-old beef calves previously vaccinated with a combination IN/parenteral MLV vaccine protocol at birth and 2 months of age or IN MLV vaccination at 2 months of age.(273, 321). In the present study, the mean SNA titers against BRSV and BHV-1 28 days following IN MLV vaccination and/or simultaneous challenge with BRSV and BHV-1 were similar among vaccinated and control calves, and significant effects of primary or booster IN MLV vaccination 2 days prior to viral challenge were not detected. Previous studies have demonstrated a reduction of BRD treatments during the first weeks of feeding in beef calves with high levels of SNA against viral pathogens

at feedlot arrival. (330-332) Therefore, vaccination protocols that do not result in high levels of SNA at the time of exposure to viral respiratory pathogens or that fail to induce rapid anamnestic immune responses may not be optimal for weaned beef calves undergoing several transport and commingling events during the marketing process.

In a previous report, a greater rate of mortality rate due to *Mh*-associated pneumonia was detected in unvaccinated 6-month-old beef steers previously challenged with BHV-1.(321) Our study demonstrated a high detection rate of *Mh* and *Pm* in nasal secretion samples from calves across treatment groups following transport, IN MLV vaccination, and experimentally infection with BRSV and BHV-1. The peak of detection of *Mh* and *Pm* occurred on day 7 after challenge. Although we did not observe mortality during the 28-day post-challenge period and did not evaluate pathological lesions, a high level of agreement between the detection of *Mh*, *Pm*, and *Mb* in nasal secretion and transtracheal wash samples from calves with clinical respiratory disease was previously reported.(259) Therefore, it is possible that in this study the presence of *Mh* and *Pm* in nasal secretion samples was a reflection of their presence in the lower respiratory tract of our study calves. Previous studies have well documented the role of an initial BRSV or BHV-1 infection on the severity of clinical disease and lung pathology in *Hs* and *Mh* pneumonia in calves. (155, 317, 318) In this study, factors such as transport and IN MLV vaccination could have been associated with the increased rate of detection of *Mh* and *Pm*. Results from one study demonstrated that a transportation event increases the detection of *Mh, Pm,* and *Mb* in nasal secretion samples of beef steers following arrival to the feedlot.(333) Additionally, results from a recent study demonstrated an increase in the detection rate of *Hs* during the first 28 days following feedlot arrival in nasal secretion samples from multi-sourced high risk beef calves previously vaccinated with an IN MLV vaccine.(156) The authors suggested that the BRSV

component of the IN MLV vaccine could have altered the nasal microbiota of vaccinated calves favoring the colonization and growth of *Hs*. In contrast, calves in our study remained negative for *Hs* and *Mb* in nasal secretion samples after transport, before IN MLV vaccination, and following IN MLV vaccination and experimental challenge with BRSV and BHV-1. *H. somni* is considered part of the commensal microbiota of the upper respiratory and genital tracts of ruminants; however, its clinical importance in cattle production systems has been mostly associated with farms located in Canada.(334, 335) Therefore, it is possible that other factors such as commingling with cattle from different origins and geographic location have an effect on the presence and growth of *Hs* in the upper respiratory tract of cattle.

Important limitations of this study include the small sample size, the lack of commingling of calves with cattle from different sources to replicate typical conditions of the southeastern U.S. beef industry, the potential infection and transmission of BHV-1 and BRSV to control calves before experimental infection and the lack of collection and testing of lower respiratory tract samples with virological, bacterial and immune response assays. It is possible that with 30 animals the power of this study was not enough to demonstrate significant differences in clinical outcomes following experimental challenge of calves. Commingling could have impacted clinical outcomes and resulted in additional changes on the respiratory tract microbiota providing valuable information. Regardless of the origin and time of occurrence, BHV-1 and BRSV infection and transmission before experimental infection in study calves could have negatively affected our ability to evaluate the effect of vaccination protocols in this study. Evaluating the presence of pathogens and immune responses in lower respiratory tract samples could have allowed a thorough evaluation of viral tropism, replication and differences in anatomic location changes in immune responses.

Conclusions

Results from previous studies have demonstrated some degree of clinical advantage of using combination vaccine protocols with parenteral and IN MLV vaccines in beef calves at different ages or stages of production such as birth, 1-2 months and 6 months (weaning time) within experimental challenge models with BRSV and BHV-1; (273, 321) however, vaccine efficacy studies that use experimental viral infection models usually cannot replicate conditions typical of natural occurrence of disease in a determined population of cattle. Factors such as commingling and geographic location could influence the presence of respiratory pathogens in the upper and lower respiratory tract and play a significant role in clinical outcomes. Additionally, based on the results of this and previous studies the use of combination vaccine protocols using IN MLV vaccines either as primary vaccination or booster of weaned (6-months-old) beef calves exposed to respiratory viruses shortly before or after vaccination may not result in prompt induction of protective local immune responses that prevent clinical disease; however, Therefore, these vaccination protocols may not be optimal for the reduction of respiratory disease triggered by early exposure to respiratory viruses in highly stressed weaned beef calves marketed through auction barns as is typical in the southeast U.S. beef industry.

Chapter 8: Conclusions

The information generated through the research performed within this program suggest that the presence of colostrum-derived SNA titers and BRSV IgG1 transferred to the upper respiratory tract of newborn beef calves likely play a role in clinical protection against clinical disease (morbidity) early in life; however, the presence of systemic and local colostrum-derived antibodies interfere with the induction of adequate/complete immune responses to IN MLV vaccination during the first month of life. It is possible that the effect of systemic and local colostrum-derived BRSV immunity on reducing BRSV morbidity and viral shedding is unremarkable following the first month of life.

Modified-live virus IN vaccination of neonatal calves and before complete transfer of specific BRSV antibodies from colostrum failed to prime significant nasal BRSV IgA responses following vaccination. Additionally, IN MLV vaccination of neonatal calves was not advantageous from the clinical perspective when BRSV was endemic in the herd where the vaccine was tested. It is possible that in cases of repeated exposure of young calves to BRSV due to endemic conditions or re-entry of the virus through new arrivals, upper respiratory tract IgA responses may be high and may provide clinical protection without the need of IN MLV vaccination. Based on the results of our studies, it is possible that young calves from farms where BRSV is not endemic could benefit from parenteral or IN MLV vaccination after the 1st month of life to avoid the interference of local and systemic maternally derived immunity.

Primary vaccination or booster with an IN MLV vaccine at weaning (6 months of age) did not provide clinical advantages nor resulted in SNA response differences following BRSV and BHV1 challenge of calves shortly (2 days) after IN vaccination. The upper respiratory tract BRDC-associated bacteria were altered by either the stress of weaning and transport, IN MLV

vaccination, and/or simultaneous experimental challenge with BRSV and BHV1. An increased detection of *Mannheimia haemolytica* and *Pasterella multocida* in nasal secretions in all calves was observed around 7 days following BRSV and BHV-1 vaccination/challenge; however, different from results of previous studies *Histophilus somni* was not detected at any time point following IN MLV vaccination and viral challenge.

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