Priming antibody responses against BRSV induced in beef calves through early vaccination

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Abstract

Bovine respiratory syncytial virus (BRSV) plays an important role in the presentation of bovine respiratory disease (BRD) in young beef calves; however, current vaccination protocols against BRSV have not significantly reduced BRD-associated morbidity and mortality. The objective of this study was to evaluate serum and nasal secretion antibody responses to BRSV in beef calves following vaccination with two different protocols.

Within 24-hours-of-life, sixty purebred beef calves were assigned to one of three different treatment groups. Group IN VAC received an intranasal (IN) modified-live (MLV) BRSV vaccine at birth and a subcutaneous (SQ) MLV BRSV vaccine at 2-months. Group SQ VAC received a SQ MLV BRSV vaccine at birth and an IN MLV BRSV vaccine at 2-months while group NO VAC was not vaccinated at birth and received an IN MLV BRSV vaccine at 2-months-of-age. Nasal secretion and serum samples were collected for assessment of BRSV antibodies prior to vaccination at <24 hours, 2-months, and 6-months-of-age. The titers of BRSV antibodies in nasal secretions and serum were similar among treatment groups. At birth, the mean log-transformed BRSV nasal IgA titers were negligible in all groups. At 2-months-of-age, the mean log-transformed BRSV nasal IgA titers in IN-SC VAC, SC-IN VAC, and NO-IN VAC calves were 192.84, 224.49, and 114.71, respectively. At 6-months-of-age, the mean log-transformed BRSV nasal IgA titers in IN-SC, SC-IN, and NO-IN calves were 178.84, 159.33, and 266.62, respectively; however, statistical significance in BRSV IgA titers was not detected among treatment groups at any time point. The model-adjusted mean-Log_2 serum BRSV antibody titers were similar among calves from all treatment groups at each sampling point. Early vaccination with MLV BRSV vaccines in a combination protocol at birth and at 2-months-

of-age was not different from single IN MLV vaccination at 2-months-of-age inducing nasal or serum BRSV-specific antibody responses in beef calves under the conditions of this study.

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Chapter 1 - Literature Review

Introduction

Bovine respiratory disease complex (BRDC) causes a significant monetary impact on the beef industry. In 2015, respiratory disease resulted in the highest percentage of nonpredatory death loss in beef cows (23.9%) and calves (26.9%) in the United States (USDA, 2015). This was followed by unknown death (14%) and old age (11.8%) in cows and calving-related issues (17.8%) and diarrhea (15.4%) in calves (USDA, 2015). For feedlots and stocker producers alone, respiratory disease resulted in 55% of death (USDA, 2015). In the state of Kansas, respiratory disease resulted in 50.3.% of nonpredatory deaths in all cattle (USDA, 2015). The cost of treating calves for respiratory disease has ranged from $23.60 per calf in beef feedlots in 2014 (Grissett et al, 2015) to an average of $42.15 per calf in California dairies in 2019 (Dubrosvky et al, 2020). This results in an estimated $800-900 million loss yearly from treatment, reduced feed efficiency, decreased rate of gain, and death in the United States (Johnson and Pendell, 2017).

BRDC is a multi-factorial disease that results from a combination of bacterial and viral pathogens, changes in the host immune system, and environmental factors. Bacterial pathogens that have been implicated include *Mannheimia haemolytica, Pasteurella multocida, Histophilus somni,* and *Mycoplasma bovis*. Viral pathogens associated with respiratory disease include bovine herpesvirus-type 1 (BHV1 or IBR), bovine respiratory syncytial virus (BRSV), bovine viral diarrhea virus 1 (BVDV 1), bovine viral diarrhea virus 2 (BVDV 2) parainfluenza virus 3 (PI3), and bovine coronavirus (BCoV).

Bovine Respiratory Syncytial Virus

Etiology and epidemiology

Bovine respiratory syncytial virus (BRSV) is a single-stranded, enveloped RNA virus of the family *Pneumoviridae* and genus *Orthopneumovirus* (Woolums, 2020). BRSV is termed a syncytial virus because of the cytopathic effect within the lung parenchyma that results in syncytial cell formation. The existence of BRSV was first suggested in 1968 when it was noted that samples of bovine serum contained neutralizing antibodies against human respiratory syncytial virus (HRSV) growth (Sacco et al, 2014). It was later termed BRSV when the first case was isolated from cattle with respiratory disease in Switzerland in 1970 (Baker et al, 1986 (3)). BRSV was first identified in the United States in Iowa and Missouri in 1974 (Baker et al, 1997).

Seroprevalence of BRSV among farms varies throughout the world. Seroprevalence among beef and dairy herds in North America ranges from 60-80% in the United States, 22-53% in Canada, and 52-91% in Mexico (Baker et al, 1997; Woolums, 2020; Figueroa-Chavez et al, 2012). In Sweden herd-level seroprevalence of BRSV range from 40-100% while Denmark has a reported herd-level seroprevalence of approximately 54% (Beaudeau et al, 2010; Ohlson et al, 2013; Uttenthal et al, 2000). Seroprevalence in cattle in South African feedlots have been reported to be approximately 43% while those in the United States are as high as 95% (Van Vuuren, 1990; Collins et al, 1988 (2)). While morbidity rates as high as 80% have been reported, mortality rates are generally low at ~2-3%; however, mortality rates as high as 20% have been reported in single outbreaks (Gershwin, 2008; Woolums, 2020).

Outbreaks of respiratory disease associated with BRSV commonly occur during the fall and winter months; however, cases have been noted during the summer months as well (Baker et al, 1997). Movement of cattle, crowding, and fluctuations of temperature may result in outbreaks in feedlot cattle (Baker et al, 1997). In young calves, BRSV outbreaks have been associated with failure of passive transfer of immunity, rapid decay of maternal antibodies, introduction of new calves into the herd, and animals that are persistently infected with BRSV within the herd (Kimman et al, 1988; Baker et al, 1986 (1); Woolums, 2020).

Cattle are the primary reservoir of BRSV. Historically, adults were believed to be the main reservoir for transmission within the herd as a result of subclinical infection and spreading BRSV to calves and younger cattle through respiratory secretions (Woolums, 2020). Results from one study suggest transmission among seropositive dairy cattle to be unlikely as it would result in long periods of disease extinction within the herd and prolonged periods of infectiousness among cattle (de Jong et al, 1996). Instead, the authors propose that calves become persistently infected with BRSV resulting in periodic shedding of the virus (de Jong et al, 1996). While the virus is typically cleared from respiratory tract epithelial cells 2-3 weeks after infection, BRSV has been shown to persist at low levels in tracheobronchial and mediastinal lymph nodes of calves for up to 71 days post-inoculation (Valarcher et al, 2001). BRSV persistence has also been demonstrated *in vitro* in B lymphocytes for up to 6 months post-infection (Valarcher et al, 2001).

Antigenic variation

Like many other viruses, BRSV has evolved its pathogenicity as a result of antigenic variation. Bovine respiratory syncytial virus is comprised of 11 proteins which includes 3 transmembrane glycoproteins within the envelope, 3 proteins that form the nucleocapsid, 3 matrix proteins, and 2 non-structural proteins (Valarcher and Taylor, 2007; Gershwin, 2008). The glycoproteins that form the envelope are the G protein, the fusion (F) protein, and the small hydrophobic (SH) protein (Gershwin, 2008). The G protein is the largest of the three consisting of 260 amino acids. The G protein facilitates attachment of the virion by the interaction of heparin-binding domains on the G protein surface and glycosaminoglycans on cell membranes (Teng et al, 1998). It has been suggested that the secreted form of the G protein may bind to neutralizing antibodies in the host (Valarcher and Taylor, 2007). The G protein may also facilitate infection by interacting with the CX3C chemokine receptor (CX3CR1), along with L-selectin (CD62L), annexin II, and surfactant proteins which in turn facilitates entrance of the nucleocapsid into the host cell (Tripp et al, 2001; Malhotra et al, 2003; Ghildyal et al, 1999).

The F protein facilitates fusion of cells and syncytia formation allowing for further spread of the virus by delivering the nucleocapsid to the cytoplasm (Gershwin, 2008). It also induces neutralizing antibodies against the virus which participate in controlling BRSV spread and prevent re-infection (Taylor et al, 1992; Taylor et al, 1995). Cleavage of the F protein to F1 and F2 subunits results in release of a 27 amino acid peptide that acts as a virokinin in smooth muscle (Gershwin, 2008). The virokinin produced results in bronchioconstriction when acting on the tachykinin receptor 3 (Zimmer et al, 2003). Secretion of this virokinin in high concentrations at the infection site may lead to desensitization of tachykinin receptors and decrease the local immune response (Zimmer et al, 2003). Cattle experimentally infected with BRSV that either contains mutations in the FCS-2 cleavage site, or are deficient in virokinins, had less inflammation and fewer histopathologic changes in the lung than those infected with wild-type strains (Valarcher et al, 2006).

The glycoproteins that form the nucleocapsid are the nucleocapsid (N) protein, phosphoprotein (P), and the RNA-dependent RNA polymerase (L) (Gershwin, 2008). The N protein is recognized by $CD8^+$ T cells which induces protective immunity against BRSV when vaccines including the N protein are administered (Gaddum et al, 2003; Taylor et al, 1997).

While both the N and P proteins play a role in regulating transcription and replication, it is the RNA-dependent RNA polymerase L protein that is responsible for performing the transcription and replication of BRSV within the cell (Yunus et al, 1998).

BRSV consists of 3 matrix proteins – M, M2-1, and M2-2. The M protein is located on the inner surface of the envelope and plays a role in the formation of virus-like particles (Teng and Collins, 1998). The M2-1 protein promotes elongation of transcription chains and increases the frequency of readthrough at gene junctions (Hardy and Wertz, 1998). The M2-2 protein then mediates the switch from transcription to replication of RNA (Bermingham and Collins, 1999). In general, BRSV matrix proteins contribute to the formation of new virions within the cell.

A major difference in paramyxoviruses like BRSV and other viruses in the family *Paramyxoviridae* is the presence of non-structural proteins NS1 and NS2 (Valarcher and Taylor, 2007). In HRSV, NS1, and to a lesser extent NS2, acts as an inhibitor of the host cell's RNA transcription and replication (Atreya et al, 1998). Both NS1 and NS2 play a role providing BRSV resistance to interferon alpha and beta, which usually activate intracellular antiviral mechanisms in the host cell. Of the proteins encoded by BRSV, the G, F, and N proteins appear to have continuously evolved since 1967 – likely due to response to vaccination (Valarcher et al, 2000).

Along with the 11 different proteins encoded by BRSV, phylogenic analysis by Elvander and colleagues in 1998 revealed 2 branches (I and II) with 4 individual subgroups (A, B, AB, and untyped) (Elvander et al, 1998). Subgroup A belongs to branch Ia while subgroup AB (comprising strains of European origin) belongs to branch Ib. Branch II includes all isolates of subgroup B (Elvander et al, 1998). More recent studies have classified the G protein in six subgroups depending on geographic location (Valarcher et al, 2000; Valentova et al, 2005).

Subgroup I include European strains identified prior to 1976 (Elvander et al, 1998; Valarcher et al, 2000; Valentova et al, 2005). Strains in subgroup II have been isolated from northern Europe, Denmark, and Sweden (Valarcher et al, 2000). Strains identified in the United States consist of subgroup III while those strains identified in the Netherlands, Belgium, and France make up subgroups II, IV, V, and VI (Valarcher et al, 2000; Valentova et al, 2005). That same study noted that animals infected with BRSV strains consisting of subgroups V and VI were more likely to experience vaccine failure (Valentova et al, 2005).

Pathogenesis

BRSV is transmitted via contact with infected cattle that have aerosolized the virus (Woolums, 2020). Fomites such as bottles and buckets may also be means of transmission of BRSV, as HRSV has been shown to be transmitted via that route (Woolums, 2020). The incubation period for BRSV is relatively short, ranging from 3-5 days after inoculation (Gershwin, 2012; Woolums, 2020). Infection with BRSV can result in bronchitis, bronchiolitis, alveolitis, or interstitial pneumonia (Kimman et al, 1989 (1); Caswell and Williams, 2016; Woolums, 2020). Clinical disease is most commonly seen in calves less than 6 months of age; however, reinfection later in life may occur and severe disease may be seen in adult cattle (Guzman and Taylor, 2015; Caswell and Williams, 2016; Woolums, 2020).

In humans, infection with HRSV results in activation of NF-κB inducing chemokines and cytokines (Harrison et al, 1999; Miller et al, 2004). A similar event occurs with BRSV infection. There is an upregulation of pro-inflammatory chemokines and cytokines in the bovine lung including IL-12, IFNγ, TNFα, IL-6, IL-18, IL-8, RANTES (CCL5), MCP-1, MIP-1α, IFNα, and IFNβ (Valarcher and Taylor, 2007; Gershwin, 2012). This upregulation is mediated by interaction of the F protein and toll-like receptor 4 (TLR4) and by the interaction of dsRNA and

TLR3 (Kurt-Jones, et al 2000; Haeberle et al, 2002; Rudd et al, 2005). In the normal lung, TLR4 is present at low levels in alveoli; however, RSV infection increases expression in epithelial cells as well as their response to TLRs (Kurt-Jones et al, 2000).

In contrast to infection with most viruses that result in activation of the JAK/STAT pathway by IFNα and IFNβ and the creation of an antiviral state, BRSV and HRSV are resistant to their antiviral effects (Schlender et al, 2000). This ability to regulate the response to IFN is mediated by the NS proteins described previously. The NS2 protein has greater inhibitory effects than NS1 when BRSV infection is present, while the opposite is true for HRSV (Valarcher et al, 2003). These NS proteins interfere with activation of IRF-3, which along with NF-κB is responsible for transcription of pro-inflammatory cytokines and chemokines (Bossert et al, 2003). Even though NS proteins are not needed for growth in vitro, infection with strains lacking either NS1 or NS2 are attenuated and when inoculated in 2-week-old calves, virus was unable to be detected 7 days post-infection (Valarcher et al, 2003).

Development of syncytia in airways and alveoli are the result of fusion of infected epithelial cells with the F protein to uninfected cells (Gershwin, 2008). After fusion, some epithelial cells undergo apoptosis (Woolums, 2020). They then slough into airways where they are phagocytized by neutrophils or alveolar macrophages (Woolums, 2020). Occasionally, BRSV will infect macrophages resulting in a decrease of Fc receptor expression, phagocytosis, phagosome-lysosome fusion, and neutrophil chemotaxis (Woolums, 2020).

Prior to the development of syncytial cells, the F protein is cleaved into F1 and F2 subunits (Zimmer et al, 2001). This cleavage allows for the release of pep27 which is later converted to virokinin (Zimmer et al, 2001; Zimmer et al, 2003). Virokinin is responsible for smooth muscle contraction and may play a role in the bronchoconstriction noted with BRSV

(Zimmer et al, 2003). This is not true for HRSV infection which suggests a species-specific modification and may be involved in eosinophil recruitment (Zimmer et al, 2003; Valarcher et al, 2006). Along with this, the F protein has been noted to result in delay in mitogen-induced lymphocyte proliferation which may influence the development of BRSV-specific memory T cells and the function of $CD8^+$ T cells (Valarcher and Taylor, 2007).

As stated previously, it is believed that the G protein may participate in BRSV infection by binding and interacting with surfactant proteins and facilitating the delivery of virions into the cytoplasm (Valarcher and Taylor, 2007). In infants who have polymorphisms in the surfactant protein-A (SP-A) and SP-D genes, infection with HRSV is enhanced and more severe as the virus has one less barrier to reach the host cell (LeVine et al, 1999; Lofgren et al, 2002).

In contrast to HRSV where 37% of human monocytes can be infected, BRSV does not infect monocyte-derived dendritic cells in the bovine (Pollock et al, 2017). However, it does affect expression of MHC I, MHC II, CD80, and CD86 expression (Pollock et al, 2017). In vitro studies have shown that BRSV strains that lack the SH protein have significantly higher levels of IL1-β and TNF-α expressed in monocytes (Pollock et al, 2017). In addition, those strains can more efficiently phosphorylate NF-κB p65 which affects cytokine expression and antigen presentation within the host (Pollock et al, 2017). SH deficient strains of BRSV also have higher levels of RANTES which enhances $CD8^+$ T cell function and the resulting cell-mediated immune response (Pollock et al, 2017).

While calves with BRSV are like humans with HRSV in that they develop a mixed Th1 and Th2 cytokine response to infection, calves with BRSV tend to have a stronger predilection for Th2 immune response (Antonis et al, 2003; Sacco et al, 2014). Studies have shown enhanced IL-4 and IL-13 production as early as 4 days post-infection (Miao et al, 2004). Calves experimentally infected with BRSV also tend to have an increased IgE concentration compared to non-BRSV-infected calves consistent with increased Th2 response (Stewart and Gershwin, 1989; Antonis et al, 2003). As a result of this increased Th2 response, infants and calves have been shown to be predisposed to allergies later in life (Antonis et al, 2003). Co-infection of BRSV and *Saccharopolyspora rectivirgula* results in a pulmonary hypersensitivity in cattle (Woolums, 2020). Similarly, cattle simultaneously experimentally infected with BRSV and *Histophilus somni* demonstrates increased serum IgE production (Gershwin et al, 2005; Agnes et al, 2013). In general, BRSV infection in calves results in mast cell degranulation, bronchoconstriction, and pulmonary edema (Jolly et al, 2004; Woolums, 2020).

Much like co-infection of BRSV with *Histophilus somni* results in increased IgE production compared to *Histophilus* infection alone, co-infection with *Mannheimia haemolytica, Pasteurella multocida,* or BVDV can also increase severity of disease. A 2018 study by Yaman and colleagues found that mixed infections of BRSV and *Mannheimia haemolytica* were present in 14/35 cases while mixed infections of BRSV and *Pasteurella multocida* were noted in 21/35 cases (Yaman et al, 2018). They suggested that infection with BRSV resulted in epithelial necrosis and hyperplasia in the lungs which lead to bacterial colonization (Yaman et al, 2018).

A separate study comparing *in vitro* colonization of *Pasteurella multocida* infection alone to that of colonization of *Pasteurella multocida* combined with BRSV found that *Pasteurella multocida* was 2-8 times more likely to adhere to BRSV infected cells than uninfected cells (Sudaryatma et al, 2018). Combined infections of BRSV and BVDV have been shown to significantly decrease Fc receptor expression, phagosome-lysosome fusion, and chemotactic factor secretion by alveolar macrophages (Liu et al, 1999). Co-infection of BVDV and BRSV also significantly decreases superoxide anion production by affected macrophages compared to either BRSV or BVDV infection alone (Liu et al, 1999).

Along with cell-mediated immunity, humoral immunity plays an important role in defending against BRSV infection in the host (Kimman et al, 1986; Kimman et al, 1987). Maternal antibodies have been shown to provide a level of protection against severe infection with BRSV (Patel and Didlick, 2004; Ellis et al, 2010). A positive correlation between high serum virus neutralizing antibodies and colostrum has been described (Chamorro et al, 2016). BRSV-specific IgM and IgA antibodies can be detected as early as 8 days post-infection in serum and nasal secretions (Kimman et al, 1987). Along with this, IgG1 can be detected at 13 days post-infection while IgG2 has been reported to take up to 3 months post-infection before it is detected in serum (Kimman et al, 1987).

Clinical signs

Clinical signs associated with BRSV infection can range from subclinical to severe. Subclinical disease may be limited to the upper respiratory tract, while clinical disease typically involves both upper and lower respiratory tracts. Calves that show clinical disease may exhibit decreased feed intake, depression, fever of 104^{o}-108^{o} F (40^{o}-42.22^{o} C), tachypnea, nasal and ocular discharge, cough, or ptyalism (Verhoeff et al, 1984; Baker et al, 1986 (2); Caswell and Williams, 2016; Klem et al, 2019; Woolums, 2020). Decreased milk production has been reported in lactating animals (Baker et al, 1997). If disease is severe, dyspnea may be pronounced (Ellis et al, 2013; Woolums, 2020).

Increased bronchovesicular sounds may be noted on thoracic auscultation (Verhoeff et al, 1984; Baker et al, 1986 (1); Woolums, 2020). Presence of crackles or wheezes secondary to pulmonary edema and emphysema may also be noted, particularly in the middle and caudodorsal lung fields (Verhoeff et al, 1984; Gershin, 2012; Woolums, 2020). Bullae may develop in the dorsal lung fields, rupture of which can result in the formation of pneumothorax; otherwise, traveling of free air from ruptured bullae can reach the mediastinum and pass into the subcutaneous spaces resulting in subcutaneous emphysema (Peek et al, 2013; Woolums, 2020).

Clinical disease can last for 1-2 weeks. Although not a consistent finding, biphasic clinical disease has been described (Sacco et al, 2014; Woolums, 2020). Cattle in the first phase present with mild to severe clinical disease. These calves overcome the disease and remain asymptomatic for days to weeks before developing peracute severe respiratory distress. This is believed to be due to either a hypersensitivity reaction or T2 helper cell response in the lower respiratory tract (Jolly et al, 2004). This peracute response is often fatal (Jolly et al, 2004).

Diagnosis

There are several ways that BRSV can be detected. Antemortem diagnostics include identification of BRSV in nasal secretions, transtracheal washes, and bronchioalveolar lavages. Specific antibodies against BRSV can be identified in serum and milk. Identification of BRSV-specific antibodies in serum can be performed by either enzyme-linked immunosorbent assay (ELISA) or serum neutralization (SN). Results from a 1986 feedlot study that compared SN antibody titers at arrival and 28 days after arrival between BRD-treated and control calves found a 62.1% BRSV sero-prevalence at arrival among both treatment groups (Martin and Bohac, 1986). One hundred percent (32/32) of calves in the BRD-treatment group with no antibodies on arrival had seroconverted by 28 days (Martin and Bohac, 1986). In the same study, of the 30/94 calves in the control group that were negative for BRSV on SN at arrival, 29 seroconverted by 28 days. Between the two groups (BRD-treated and control), 71.3% of calves were positive for BRSV-specific SN antibodies 28 days after arrival to the feedlot by seroconversion.

Additionally, the estimated incidence of BRSV infection was 100% and the calves that seroconverted were 2.4 times more likely to be treated for BRD compared to seropositive calves.

Detection of serum antibodies by ELISA has been performed on both pre-weaning and weaning beef and dairy calves. In 1998, Graham et al compared the use of ELISA for detection of serum antibodies for BRSV, BVDV, IBR, and PI-3 to that of virus neutralization and hemagglutination inhibition. A total of 564 paired sera from 145 outbreaks of respiratory disease were analyzed (Graham et al, 1998). The highest incidence of seroconversion was to BRSV at 19%, followed by PI-3 at 13.7% (Graham et al, 1998). Of the 195 animals that seroconverted, 25% exhibited seroconversion to more than 2 viruses tested (Graham et al, 1998). They determined that ELISA had a 95% specificity and 92% sensitivity for the detection of BRSV-specific antibodies in serum when compared to virus neutralization, indicating that it is an effective way to determine seroconversion in cattle (Graham et al, 1998).

A 2011 study followed 10 Holstein calves aged 9 to 32 days over 6 weeks and evaluated the presence of acute phase proteins (APP), serum haptoglobin, lipopolysaccharide binding protein (LBS), serum amyloid A (SAA), and alpha$_1$-acid glycoprotein (AGP) in calves with BRD secondary to BRSV (Orro et al, 2011). Serum BRSV-specific IgG1 concentrations were determined by ELISA while the presence of BRSV in BAL was determined by nested RT-PCR (Orr et al, 2011). Calves were classified as being either low response or high response, based on the presence of detectable BRSV-specific IgG1 at 4 weeks and an optical density of >1.5 at 6 weeks (Orro et al, 2011). At 2 weeks 50% of the calves (2 low response, 3 high response) were PCR positive for BRSV on BAL samples (Orro et al, 2011). While LBS, SAA, and AGP were elevated in the first 2 weeks, LBS and SAA increased in the low response group at 2-3 weeks and remained elevated through the sampling period (Orro et al, 2011). This study showed that

SAA and LBS are sensitive markers of respiratory infection, but because SAA and LBS were not measured in healthy cattle, specificity could not be determined (Orro et al, 2011).

A 2014 study by Chamorro and colleagues evaluated the difference in BRSV serum antibodies in at 2 days, 2 months, and 5 months of age calves that were fed maternal colostrum versus those calves that were fed colostrum replacer (Chamorro et al, 2014). Two of the three farms routinely administered pre-breeding vaccinations that included BRSV antigens (Chamorro et al, 2014). They found there was a greater variance in titers among calves that were fed maternal colostrum compared to colostrum replacer-fed calves, both groups had a significant decrease in BRSV-specific antibodies as calves aged and maternal antibodies waned (Chamorro et al, 2014).

Detection of BRSV in nasal secretions has been performed using ELISA, rt-PCR, Nano-PCR, and rapid test strips designed for HRSV in humans. Utilization of ELISA for detection of BRSV-specific antibodies in nasal secretions relies on detection of IgG, IgG1, IgG2, and IgA (Ellis et al, 2018 (1)). Determination of antibody titers is made by evaluation of optical density from serum of affected animals in comparison to that of naturally exposed cattle. A 2018 study evaluating the presence of IgG1 and IgG2 in nasal secretions of 17 Holstein calves that received colostrum replacer found negligible levels of IgG1 and IgG2 prior to colostrum and negligible IgG2 concentrations after colostrum intake (Ellis et al, 2018 (1)). In contrast, there was a significant increase in IgG1 after colostrum was consumed (Ellis et al, 2018 (1)).

A 2017 study by Doyle and colleagues compared four different sampling techniques for the detection of *Pasteurella multocida, Mannheimia haemolytica, Mycoplasma spp., Mycoplasma bovis*, BRSV, and BCoV by rt-PCR or culture (Doyle et al, 2017). Samples evaluated included nasal secretions (NS), deep nasopharyngeal swabs (NPS), transtracheal wash

(TTW), and bronchoalveolar lavage (BAL) (Doyle et al, 2017). One hundred Holstein and Holstein-cross bull calves were enrolled in the study based on the presence of respiratory disease with a score greater than or equal to 5 as identified by the Wisconsin calf respiratory scoring chart and a rectal temperature >103° F (University of Wisconsin, 2020; Doyle et al, 2017). All calves were over 30 days old at the time of enrollment. Of the 100 calves that were enrolled in the study, 94 of 98 were tested for BRSV using one or more of the sampling locations and methods (Doyle et al, 2017). Nine of Ninety-six (9.4%) calves were rt-PCR positive for BRSV on nasal secretions while 13/94 (13.8%) were positive via deep nasopharyngeal swab (Doyle et al, 2017). Bronchoalveolar lavage and transtracheal wash had similar results at 15/94 (16%) and 17/98 (17.4%) respectively (Doyle et al, 2017). After determining positivity, results from nasal secretions, nasopharyngeal swabs, and bronchoalveolar lavages were compared to those of the transtracheal washes. The agreement between TTW and BAL was very good at 92.9%, while NS was moderate at 60.9% and NPS was good at 78.6% (Doyle et al, 2017). These findings contrasted with BCoV, which had significantly higher proportions of positive results from NS (15.6%) and NSP (20.9%) when compared to TTW (6.6%) (Doyle et al, 2017). A similar study performed previously that evaluated NPS in 2,014 calves found 20% were positive for BRSV (Niebergs et al, 2014).

A study comparing rt-PCR and immunofluorescence (IF) on NPS samples found that 31/35 (89%) of isolates were rt-PCR positive for BRSV while IF only detected BRSV in 23 (66%) of cases (Vilcek et al, 1994). Those 31 cattle positive on rt-PCR exhibited clinical disease at time of sampling while the 4 negative cattle were asymptomatic (Vilcek et al, 1994). Seroconversion was detected in all 35 head by ELISA at a second sampling 3 weeks later (Vilcek et al, 1994). The authors also noted that the nested PCR was 10 times more likely to determine positivity than single PCR or virus isolation (Vilcek et al, 1994).

Along with the recent identification of a nano rt-PCR for identification of BRSV in nasal secretions, two different studies have been performed to evaluate the use of different rapid human respiratory syncytial virus strip tests for the detection of BRSV in both apparently healthy and experimentally vaccinated calves (Socha and Rola, 2015; Urban-Chmiel et al, 2015). These strips detect HRSV viral antigens through immunochromatography at the patient side. The first study compared the use of RSV Respi-strip, TRU RSV, and Binax-NOW RSV strips to rt-PCR on nasal secretions and serum antibody titers as determined by ELISA (Socha and Rola, 2015). Five, 6-8-week-old BRSV seronegative calves were enrolled in the study. Three calves were administered an intranasal vaccine containing BRSV and PI3 (Risposal RS-PI3) after serum was collected (Socha and Rola, 2015). A total of 10 nasal swabs were then performed on each calf over a 28-day period (Socha and Rola, 2015). All 3 test strips and the rt-PCR reacted positively to the two different BRSV strains and single HRSV strain used as positive controls (Socha and Rola, 2015).

The RSV Respi-strip detected BRSV in 10/30 samples from vaccinated calves, while the TRU RSV only detected BRSV in 4/30 samples, and the BinaxNOW RSV test detected BRSV in 9/30 samples (Socha and Rola, 2015). The BinaxNOW RSV strip was revealed to have a 50% sensitivity while the other two tests only have 33% sensitivity to BRSV detection (Socha and Rola, 2015). Despite this low sensitivity, each test had a relatively high specificity of 87% for RSV Respi-strip and BinaxNOW RSV and 100% for TRU RSV (Socha and Rola, 2015). While TRU RSV had a low sensitivity at 33%, its specificity of 100%, positive predictive value possibly approaching 100% and negative predictive value of 74% may make it a viable option of rapid on-farm confirmation of BRSV infection in affected calves (Socha and Rola, 2015). However, larger sample sizes are needed to determine the true efficacy of this test (Socha and Rola, 2012).

While sampling of serum, nasal secretions, and even NPS are the most common ways of detecting BRSV antibodies antemortem, antibodies against the virus have also been detected in bulk tank and individual milk samples. Several studies have been performed that compare BRSV and BCoV antibodies in serum and milk via ELISA (Ohlson et al, 2014; Toftaker et al, 2018). A 2014 study evaluated samples from 105 cows in 27 Swedish dairies (Ohlson et al, 2014). All cows included in the study were apparently healthy, and none had been vaccinated for either BRSV or BCoV. IgG antibodies were analyzed using a commercially available indirect ELISA (Ohlson et al, 2014). Eighty of eighty-two cows that were positive for BRSV antibodies in serum were also positive in milk while 72/77 cows that were positive for BCoV in serum were positive in milk (Ohlson et al, 2014). A percent positivity of <5 gave a 100% sensitivity and 95.7% specificity (Ohlson et al, 2014). Increasing this percent positivity value to 15 decreased the sensitivity of the test to 95.1% while specificity remained unchanged (Ohlson et al, 2014). This study showed close correlation between milk and serum samples for BRSV-specific antibody detection and gives a more convenient method for surveillance of BRSV infection in unvaccinated dairy herds (Ohlson et al, 2014).

A similar study performed in 2018 compared the use of an MVD-Enferplex BCoV/BRSV multiplex against two commercially available ELISAs specific for BCoV or BRSV respectively. Bulk tank milk samples (720) were evaluated (Toftaker et al, 2018). While both the multiplex and ELISA had similar sensitivities for BRSV-specific antibodies at 94.4% and 99.8% respectively, the multiplex had a higher specificity at 90.6% while the ELISA used was only

57.4% (Toftaker et al, 2018). This specificity was increased to 99.4% with a sensitivity of 93.4% when the percent positivity was >50 (Toftaker et al, 2018).

Gross postmortem examination of cattle infected with BRSV reveals cranioventral lobules that may be dark red-purple or collapsed in areas due to atelectasis (Bryson et al, 1983; Caswell and Williams, 2016). Areas of consolidation may be noted throughout the cranial, middle, or accessory lobes (Bryson et al, 1983). The caudodorsal lungs may fail to collapse, and if emphysema is present may appear pale (Baker et al, 1986 (3); Kimman et al, 1989 (1); Woolums et al, 1999; Schreiber et al, 2000). The nasal meatus, trachea, bronchi, and bronchioles may contain foamy or mucopurulent discharge (Bryson et al, 1983; Kimman et al, 1989 (1); Sacco et al, 2014). A demarcation between emphysematous and consolidated lungs is often apparent (Verhoeff et al, 1984; Sacco et al, 2014). If bulla or emphysematous lesions have ruptured, pneumothorax, pneumomediastinum, or pneumopericardium may be present (Baker et al, 1986 (2); Kimman et al, 1989 (1); Schreiber et al, 2000). Along with this, mediastinal lymph nodes are often enlarged (Baker et al, 1986 (2); Kimman et al, 1989 (1)).

Microscopic examination reveals bronchointerstitial pneumonia, necrotizing, bronchiolitis, type II pneumocyte hyperplasia, syncytia formation, and exudative alveolitis (Bryson et al, 1983; Baker et al, 1986 (2); Kimman et al, 1989 (1); Woolums et al, 1999; Schreiber et al, 2000). Necrotizing bronchiolitis is characterized by necrotic ciliated and non-ciliated respiratory epithelium (Kimman et al, 1989 (1)). Epithelial cell necrosis leads to ulceration of the epithelial layer and flattened cells lining the bronchioles that have a squamous morphology (Bryson et al, 1983). Fibrin casts, alveolar macrophages, seroproteinaceous fluid, and occasional neutrophils may be noted in the alveoli (Bryson et al, 1983; Woolums et al, 1999; Sacco et al, 2014). Syncytial cells project from the bronchiolar wall and occasionally in the alveoli (Kimman et al, 1989 (1)). Eosinophilic intracytoplasmic inclusion bodies may be noted in syncytial cells (Baker et al, 1986 (3); Schreiber et al, 2000). Edema often results in dilated lymphatic vessels and widened interlobular septa (Bryson et al, 1983; Kimman et al, 1989 (1)). Evaluation of tracheobronchial lymph nodes reveals prominent follicles within enlarged cortices along with expanded parafollicular areas likely due to lymphocytic hyperplasia (Kimman et al, 1989 (1)). Lymphocytes, plasma cells, hemosiderin-laden macrophages, and occasional neutrophils and eosinophils may be seen in medullary sinuses (Collins et al, 1988 (1); Sacco et al, 2014).

Chronic infection may result in bronchiolitis obliterans and epithelial hyperplasia (Bryson et al, 1983; Collins et al, 1988 (1); Schreiber et al, 2000). Persistent inflammation leads to fibroblast infiltration and neovascularization (Sacco et al, 2014). If severe enough, fibrous polyps covered by respiratory epithelium may extend into the lumen decreasing airflow and ventilation in as little as 10 days post-infection (Caswell and Williams, 2016). It should be noted however, that this can occur with numerous viral, bacterial, fungal, and parasitic agents and is not a specific finding with BRSV infection (Bryson et al, 1983; Sacco et al, 2014).

Detection of BSRV on lung tissue can be performed using rt-PCR, immunohistochemistry (IHC), immunofluorescent antibody (IFA) staining, and direct fluorescent antibody test (d-FAT) (Verhoeff et al, 1984; Kimman et al, 1989 (1); Woolums et al, 1999; Timsit et al, 2010; Caswell and Williams, 2016; Kamdi et al, 2020; Woolums, 2020). IHC is unique in that it can be performed on both fresh, frozen, and formalin-fixed tissues. A recent study of 406 cattle and buffaloes in India noted a strong correlation between rtPCR and d-FAT results (Kamdi et al, 2020). One hundred percent (12/12) of cattle positive for BRSV on rt-PCR were positive when using d-FAT indicating this is a potential alternative for detection on paraffin-embedded tissue samples (Kamdi et al, 2020).

Vaccination

The mainstay of BRSV outbreak prevention is vaccination. Most clinical disease is noted in calves 2-6-months of age, and vaccination prior to weaning is often recommended. The problem with vaccination of young calves, is that maternal antibodies against BRSV can interfere with vaccine response and efficacy (Ellis et al, 2014). Reports in humans suggest that vaccination with formalin inactivated HRSV vaccine will not only fail to provide adequate protection against RSV but may increase severity of respiratory disease (Kapikian et al, 1969). Similar findings were demonstrated by Schreiber and colleagues in 2000, when they described a BRSV outbreak with high mortality rates in Belgian Blue cattle that had previously been vaccinated with a β-propiolactone-inactivated BRSV-vaccine (Schreiber et al, 2000).

Both parenteral and intranasal vaccines have been available since the 1980s. Most of these vaccines are in combination with IBR, PI3, and BVDV types 1 and 2. The efficacy of administration of modified-live BRSV vaccines in reduction of naturally acquired or induced disease has not been shown, with a recent systematic review indicating that published studies failed to suggest a difference in morbidity and mortality between vaccinated and unvaccinated calves (Theurer et al, 2015). Most vaccinated calves will shed the virus; however, the peak titer is suppressed in vaccinated calves when compared to unvaccinated controls (West et al, 1999). Clearance of the virus after vaccination coincides with mucosal antibody formation, the presence of cytotoxic T cells within the lungs, and serum antibody responses (Ellis et al, 2001).

Previous studies performed using parenteral vaccines suggested they did not elicit IgA or serum antibody responses in calves where maternal antibodies were still present (Fulton et al, 2004; Ellis et al, 2014). A 2014 study performed by Ellis and colleagues evaluated the effects of administration of a 5-way vaccine containing BRSV (Vista 5®) or a 3-way vaccine without BRSV (Vista 3®) on 34 Holstein calves at 3-9-days of age (Ellis et al, 2014). No calf was shedding BRSV in nasal secretions prior to vaccination and no significant difference was noted among treatment group in serum IgG or nasal secretion IgA concentrations (Ellis et al, 2014). In addition, there was no IFNγ or IL-4 production detected in the plasma of any calf vaccinated against BRSV (Ellis et al, 2014). The authors found an apparent inhibition of acquired immunity in calves in the face of maternal antibodies (IFOMA) (Ellis et al, 2014). There was no evidence of humoral or cell-mediated responses to vaccination in contrast to previous studies with similar vaccination histories (West et al, 2000; Ellis et al, 2001; Ellis et al, 2013).

Evaluation of seroconversion as evidenced as 4-fold-increases in serum titers is commonly used to determine vaccination response. As stated, vaccination IFOMA does not often result in seroconversion, but reports of an anamnestic response following vaccination with a second dose of a modified-live vaccine have been made. This response can be as long as 112-123 days after vaccination (Kaeberle et al, 1998; Kirkpatrick et al, 2008). Because of this delayed response, age at vaccination and level of maternal antibodies may influence antibody response in calves. Reports of maternal antibody titers against BRSV as low as 32 are able to interfere with seroconversion when killed vaccines are administered (Chamorro et al, 2014).

A 1999 study evaluating the efficacy of modified-live vaccines in calves experimentally challenged with BRSV found that vaccinated calves had significantly less clinical disease, hypoxemia, nasal shedding of virus, and lung lesions noted on necropsy (West et al, 1999). They noted that protection was associated most closely with the presence of IFNγ and MHC1-restricted cytotoxic T cells (West et al, 1999).

Several studies were performed in the 2000s evaluating a modified-live combination vaccine containing BRSV, IBR, PI3, and BVDV1 (Rispoval 4®) (Peters et al, 2004; Harmeyer et al, 2006; Salt et al, 2007). Peters et al, 2004 reported at study that involved Rispoval 4® administered to calves 7-weeks to 6-months old twice at 3-week intervals (Peters et al, 2004). Calves were challenged 6-12 months after vaccination (Peters et al, 2004). While there was no difference in clinical signs, with both groups developing nasal discharge and pyrexia, calves that were vaccinated had much higher levels of antibodies to BRSV than unvaccinated calves and viral shedding was decreased from 3.4 to 1.2 days (Peters et al, 2004).

A similar study performed in 2007 evaluated the administration of Rispoval 4® to calves 2-9-months-of-age using two doses 3-weeks apart (Salt et al, 2007). Calves were then challenged at 3-5 weeks post-vaccination (Salt et al, 2007). Vaccinated calves had enhanced antibody responses and decreased viral shedding when compared to unvaccinated controls (Salt et al, 2007). Decreased shedding was associated with a significant elevation in serum IgG as determined by ELISA. There was no significant difference in clinical disease (Salt et al, 2007).

Because concerns regarding decreased antibody response after parenteral vaccination IFOMA, vaccination with modified-live intranasal (IN) vaccines has been advocated (Ellis et al, 2007). Intranasal inoculation against BRSV was first suggested in the late 1980s when it was discovered that IN vaccines resulted in both local IgA and systemic IgG responses after challenge (Kimman et al, 1989 (2)). Exposure of nasal mucosa to BRSV IN vaccines can override maternal antibody responses and is effective in priming the immune response in young calves (Ellis et al, 2013).

A 2004 study evaluated calves vaccinated with an IN modified-live vaccine at 4-6-weeks old (Woolums et al, 2004). Three groups of calves were evaluated – those that received the IN vaccine, those that were mock-vaccinated with spent tissue culture media IN, or unvaccinated controls (Woolums et al, 2004). Calves were challenged with BRSV at 8-weeks of age and vaccinated calves appeared to have reduced clinical disease in comparison to control calves (Woolums et al, 2004). IFNγ production was increased in lymphocytes despite lacking an amnestic response (Woolums et al, 2004).

In 2010, Ellis et al evaluated the response of seronegative and seropositive calves to experimental challenge with BRSV following IN vaccination (Ellis et al, 2010). Twenty-one 3-8-day old calves (11 seropositive, and 10 seronegative) were either administered Vista Once SQ® or distilled water intranasally in the first experiment. All calves developed respiratory disease after BRSV challenge at approximately 4.5 months of age and those in the seropositive group had a significantly higher rectal temperature than seronegative calves (Ellis et al, 2010). There was no significant difference in mortality noted between treatment groups (Ellis et al, 2010). Calves in the seronegative vaccinated group had significantly fewer days of viral shedding than either seropositive vaccinated or unvaccinated controls (Ellis et al, 2010). Only 4 calves developed an amnestic response with elevated IgG after challenge (Ellis et al, 2010). There was no significant difference found in lung lesions on necropsy of affected animals (Ellis et al, 2010). A second experiment was performed with 11 seronegative calves receiving Vista Once SQ® intranasally and 12 seronegative calves receiving the vaccine parenterally (Ellis et al, 2010). Clinical disease was not different among treatment groups. Vaccinated calves shed significantly less virus and had less severe lung lesions than unvaccinated controls (Ellis et al, 2010).

A separate 2010 study evaluated the administration of a modified-live vaccine containing BVDV types I and II, IBR, PI3, and BRSV intranasally in calves aged 3-8 days (Xue et al, 2010). Calves were challenged with BRSV 21 days post-vaccination (Xue et al, 2010).

Unvaccinated calves developed high fever and respiratory rates, dyspnea, depression, anorexia, cough, and nasal discharge 3 days post-inoculation (Xue et al, 2010). Vaccinated calves developed significantly fewer clinical signs and had lower clinical scores (Xue et al, 2010). Along with more severe disease, unvaccinated calves shed significantly more virus in nasal secretions than vaccinated calves at 4-7 days post-inoculation (Xue et al, 2010).

In 2013, Ellis et al evaluated the duration of immunity of a modified-live BRSV vaccine when administered IN to 84 calves between 3 and 11 days old (Ellis et al, 2013). Three separate experiments were performed using calves either seronegative or seropositive to BRSV. Calves were challenged at 7 weeks (experiment 1), 9 weeks (experiment 2), or 3.5 months (experiment 3) after vaccination (Ellis et al, 2013). There was no significant difference in vaccinated and unvaccinated calves regarding clinical disease; however, vaccinated calves had significantly fewer days with signs of depression (Ellis et al, 2013). Vaccinated calves in experiment 1 had significantly fewer days where virus was shed in nasal secretions (Ellis et al, 2013). There was a significant difference in mortality rate prior to euthanasia as well as lung lesions on necropsy in calves in experiments 1 and 2 (Ellis et al, 2013). The authors found a negative association between IgG and IgA titers and lung lesions in experiment 1, indicating that as antibody titers increased, lung lesions decreased (Ellis et al, 2013). Both experiment 2 and 3 showed a negative association between IgG titers and lung lesions, and experiment 3 had a positive association between IgG and PaO_2 (Ellis et al, 2013). These findings suggest that duration of immunity to BRSV after vaccination at 3 to 11 days of age is less than 4 months (approximately 14 weeks) when maternal antibodies are present, and that specific BRSV antibodies (IgG and IgA) may be associated with clinical protection after vaccination (Ellis et al, 2013).

Finally, a study was performed in 2018 to evaluate the efficacy of boosting antibody responses to BRSV through parenteral vaccination in calves that received IN vaccines early in life (Ellis et al, 2018 (2)). Seventy-five Angus-cross calves were enrolled in the study. Fifty received a single dose of IN vaccine at birth. At two-months-of-age, 24 calves were administered a modified-live vaccine and 25 calves received a killed vaccine SQ (Ellis et al, 2018 (2)). Unvaccinated calves at birth received the modified-live vaccine SQ at 2-months-of-age (Ellis et al, 2018 (2)). Calves were weaned at 6-7 months and 20 calves were selected for challenge. Eighteen of twenty calves developed mild respiratory disease 7 days after challenge (Ellis et al, 2018 (2)). The other 2 calves developed severe respiratory disease and required euthanasia. By day 14 the remaining 18 calves were considered clinically normal and returned to the herd. There was no significant difference in clinical signs among treatment groups at 6-months-of-age (Ellis et al, 2018 (2)). There were no significant differences among groups on viral shedding in nasal secretions (Ellis et al, 2018 (2)). Inactivated vaccine vaccinated calves had significantly higher PaO_2 concentrations compared to other groups (Ellis et al, 2018 (2)). All 20 calves had high BRSV-specific IgG concentrations in serum (Ellis et al, 2018 (2)). IgA antibodies were low and not significantly different among treatment groups at any time point (Ellis et al, 2018 (2)).

Some adverse reactions to vaccination against BRSV have been reported, including increased morbidity and mortality rates (Schreiber et al, 2000; Larsen et al, 2001). While it has been suggested that priming induced by vaccination with an inactivated BRSV vaccine resulted in a hypersensitivity reaction after natural exposure to disease, there is little evidence that this occurs (Schreiber et al, 2000; Larsen et al, 2001). This apparent response could have little to do with vaccination, and more to do with timing and severity of the disease present within the herd (Ellis, 2020).

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Chapter 2 - Antibody responses against BRSV induced in beef calves through early vaccination

Abstract

Bovine respiratory syncytial virus (BRSV) plays an important role in the presentation of bovine respiratory disease (BRD) in young beef calves; however, current vaccination protocols against BRSV have not significantly reduced BRD-associated morbidity and mortality. The objective of this study was to evaluate serum and nasal secretion antibody responses to BRSV in beef calves following vaccination with two different protocols.

Within 24-hours-of-life, sixty purebred beef calves were assigned to one of three different treatment groups. Group IN VAC received an intranasal (IN) modified-live (MLV) BRSV vaccine at birth and a subcutaneous (SQ) MLV BRSV vaccine at 2-months. Group SQ VAC received a SQ MLV BRSV vaccine at birth and an IN MLV BRSV vaccine at 2-months while group NO VAC was not vaccinated at birth and received an IN MLV BRSV vaccine at 2-months-of-age. Nasal secretion and serum samples were collected for assessment of BRSV antibodies prior to vaccination at <24 hours, 2-months, and 6-months-of-age. The titers of BRSV antibodies in nasal secretions and serum were similar among treatment groups. At birth, the mean log-transformed BRSV nasal IgA titers were negligible in all groups. At 2-months-of-age, the mean log-transformed BRSV nasal IgA titers in IN-SC VAC, SC-IN VAC, and NO-IN VAC calves were 192.84, 224.49, and 114.71, respectively. At 6-months-of-age, the mean log-transformed BRSV nasal IgA titers in IN-SC, SC-IN, and NO-IN calves were 178.84, 159.33, and 266.62, respectively; however, statistical significance in BRSV IgA titers was not detected among treatment groups at any time point. The model-adjusted mean-Log_2 serum BRSV antibody titers were similar among calves from all treatment groups at each sampling point.

Early vaccination with MLV BRSV vaccines in a combination protocol at birth and at 2-months-of-age was not different from single IN MLV vaccination at 2-months-of-age inducing nasal or serum BRSV-specific antibody responses in beef calves under the conditions of this study.

Introduction

Bovine respiratory disease (BRD) is the leading cause of death of beef calves between 3-weeks-of-age and weaning (USDA, 2015); however, the proportion of pre-weaning beef calves affected by BRD varies among United States (US) cow-calf farms with some reporting a prevalence >20% while others reporting none (Hanzlicek et al, 2010; Woolums, et al; 2013). Bovine respiratory syncytial virus (BRSV) is common among US beef operations with a seroprevalence ranging from 60-80% and morbidity and mortality rates as high as 80% and 20% respectively (Baker et al, 1986; Schreiber et al, 2000; Larsen et al, 2001; Gershwin et al, 2008).

Vaccination of young calves with intranasal (IN) or parenteral modified-live virus (MLV) vaccines has been adopted as the main strategy to reduce clinical disease (Ellis et al, 2007; Ellis et al, 2010; Xue et al, 2010; Ellis et al, 2014). Despite this strategy, evidence of clinical protection provided by vaccination in the face of maternal antibodies (IFOMA) has remained inconsistent (Ellis et al, 2010; Ellis et al, 2013; Ellis et al, 2018 (2), Kolb et al, 2020). Additionally, a recent meta-analysis reported that vaccination against BRSV with a MLV vaccine did not significantly decrease the risk of BRD-associated morbidity and mortality in calves (Theurer et al, 2015).

The majority of MLV BRSV vaccination protocols advocate vaccinating calves between 3 and 30-days-of-age. However, the presence of maternally derived antibodies in nasal secretions and serum during this time interferes with priming of both humoral and cell-mediated immune responses (Kimman et al, 1988; Ellis et al, 2010; Ellis et al, 2014). As a result, calves may experience decreased clinical protection. Previous reports have noted that calves vaccinated with a MLV BRSV vaccine early in life and IFOMA have a decreased duration of immunity against BRSV (Ellis et al, 2010; Ellis et al, 2013). However, vaccination prior to complete absorption of maternal antibodies may provide better priming of humoral and cell-mediated immune responses systemically and locally in the upper respiratory tract.

The objective of this study was to evaluate the effect of two different vaccination protocols administered during the first day of life and at 2-months of age on nasal secretion and serum BRSV antibody titers at 2-months and 6-months of age. We hypothesized that serum antibody titers would be similar among vaccine-treatment groups at 2-months and 6-months of age; however, differences in nasal secretion BRSV-IgA titers would be observed in different vaccine-treatment groups.

Materials and methods

Animals and experimental design

Sixty newborn beef calves from the Kansas State University Purebred Beef Unit (31 heifers and 29 bulls) representing Angus (n = 36), Simmental (n = 14), and Hereford (n = 10) breeds were enrolled in the study between January 21 and March 31, 2018. Calves were born to first-calf heifers in individual stalls and remained indoors for 24 hours after birth before being placed in a single pasture. All dams were vaccinated with a single dose of a modified-live vaccine containing bovine herpesvirus type 1 (BHV-1), bovine viral diarrhea virus (BVDV) types 1 and 2, parainfluenza virus type 3 (PI3), BRSV, *Campylobacter fetus, Leptospira canicola, L. grippotyphosa, L. hardjo, L. icterohemorrhagiae,* and *L. Pomona* (Bovishield Gold 5 FP5®)[1] 1 month prior to breeding. Calves were allowed to nurse colostrum from their dams after birth naturally and without assistance. This study was approved by the Kansas State University Institutional Animal Care and Use Committee (PRN # 4039).

The study calves were randomly assigned to one of three treatment groups using a random number generator (Microsoft Excel®)[2] by on-farm personnel at the time of birth. Within the first 24 hours of life (T1), calves in the IN-SC VAC group received a single dose (2 mL) of an IN MLV BRSV-vaccine (Inforce 3®)[3] in the right nostril. Calves in the SC-IN VAC group received a single dose (2 mL) of a parenteral MLV BRSV-vaccine (Express 5®)[4] subcutaneously (SC) over the right side of the neck. Calves in the NO-IN VAC group remained as the unvaccinated controls. At two-months-of-age (T2), calves in the IN-SC VAC group received a single dose (2 mL) of a parenteral MLV BRSV-containing vaccine (Express 5®) subcutaneously over the right side of the neck while calves in the SC-IN VAC group received a single dose (2 mL) of an IN MLV BRSV-vaccine (Inforce 3®) in the right nostril. Calves that were in the NO-IN VAC group at birth received a single dose (2 mL) of an IN MLV BRSV-vaccine (Inforce 3®) in the right nostril.

Nasal secretion and serum samples were collected at birth (0-24 hours; T1), 2-months-of-age (T2), and 6-months-of-age (T3) for the detection of BRSV serum neutralizing antibodies (Ab) and BRSV-specific IgA, respectively. Nasal secretions samples were collected using cotton plugs attached to an approximately 5cm piece of non-absorbable suture (3 Braunamid)[5]. The swabs were then soaked in sterile saline before being inserted in the left nostril for 3-6 minutes. After removal from the nares, swabs were placed in 35 mL syringes[6] and secretions were expressed into red top tubes prior to transport. Nasal secretions in 0.5 μL aliquots were pipetted into cryogenic vials[7] and placed in a -80°C freezer until the completion of the study for analysis.

All serum samples were placed in a -80°C freezer until the completion of the study for further analysis.

All study calves received 5 ml of an inactivated vaccine containing *Clostridium chauvoei, C. septicum, C. haemolyticum, C. novyi, C. sordellii* and *C. perfringens* type C and D toxoid (Ultrabac 8®)[8] subcutaneously over the left side of the neck at both T2 and T3.

Measurement of BRSV-specific IgA in nasal secretions

Bovine respiratory syncytial virus inactivated with 2 µM binary ethyleneimine followed by neutralization with sodium thiosulfate was diluted 1:800 in carbonate bicarbonate buffer (pH 9.5) and used to coat plates incubated overnight at 4°C. Plates were washed three times with PBS containing 0.05% TWEEN 20 (ELISA wash buffer). Initial processing of nasal secretions consisted of diluting them 1:1 with PBS containing 0.1% Pluronic® F-127 (Sigma), vortexing thoroughly and storing them at -80°C prior to analysis. After thawing nasal secretions were vortexed again and diluted 1:100 in ELISA wash buffer. From this initial dilution serial 2-fold dilutions were prepared out to 1:800 and each dilution was run in triplicate. Samples that exceeded the range were tested again at a higher dilution and samples that were negative were tested again at a lower dilution. In addition to the samples each plate also included the following (4 replicates each): 1) a positive control which consisted of nasal secretions diluted 1:100 that would yield an average optical density between 0.3-0.8, 2) a negative control which was low IgG FBS (Gibco) diluted 1:100 and 3) a plate blank which was ELISA wash buffer alone. The IgA was detected with commercially available HRP conjugated rabbit anti-bovine IgA (Bethyl Laboratories) diluted 1:500 and plates were developed using 2,2'-azino-bis-3-ethylbenzothiazo-line-6-sulphonic acid (ABTS, Roche) and were read on a plate reader at a wavelength of 405 nm.

Titers were reported as the inverse of last dilution which was greater than or equal to two times the average optical density of the negative control.

Measurement of BRSV-specific antibodies in serum

Virus neutralization (VN) for serum antibodies was performed as previously described (Ballew, 1986; Hoppe et al, 2018) by the Kansas State University Veterinary Diagnostic Laboratory. Serum samples were thawed and inactivated in a water bath at 56^{o}C for 30 minutes before being diluted from 1:10 to 1:1000 in 96-well flat bottom microplates with 500 µL of 100 $TCID_{50}$ BRSV suspended in minimum essential medium (MEM). Following incubation at 37^{o}C in 5% CO_2 for 1 hour, cultures were inoculated with MEM containing 7% bovine serum and antibiotics (SM Media). The plates were then incubated up to 2 weeks and monitored daily for the absence of cytopathic effect. Titers were then reported as the inverse of the lowest dilution required to inhibit cytopathic effect.

Statistical analysis

The two dependent variables for this study consisted of BRSV IgA antibody titers from nasal secretions and BRSV antibody titers from serum (log_2 transformed). Independent variables of interest included the vaccine protocol (IN-SC, SC-IN, and NO-IN), and time of sampling (less than 24 hours, 2-months-of-age, and 6-months-of-age). Two other independent variables, breed (Angus, Hereford, and Simmental) and sex (male and female), were considered potential *a priori* confounders of the association between vaccine protocol and antibody titers.

For both dependent variables, linear mixed models (LMM) were fitted using *proc glimmix* in SAS 9.4 (SAS Institute Inc., Cary, NC, USA) using a Gaussian distribution, identity link, and a restricted pseudo-likelihood estimation. A univariable screen was ran for all independent variables, against each of the dependent variables. Variable significance was assessed using a Wald test with a Chi-square distribution.

Breed and sex were evaluated for confounding by including each variable separately in a bivariable model including the variable pertaining to vaccine protocol and comparing the magnitude of its coefficients from the univariable and bivariable models, as well as its significance. If the coefficient exhibited a > 30% change or there was a significant change in *P* value, the variable was considered a confounder and evaluated in the final model. The *final model* included main effects for vaccine protocol, time of sampling and the two-way interaction term between vaccination protocol and time of sampling.

A Toeplitz covariance structure was fitted to account for repeated measures. Model assumptions of homoscedasticity and normality were visually assessed using residual plots. Log_2, $\log_{10}$, and natural log transformations of the IgA antibody titers were performed and based on AIC and BIC values, the $\log_{10}$ transformation was chosen. Model-adjusted means, 95% confidence intervals and *P*-values were also assessed. The Tukey-Kramer correction was used to adjust the *P*-value for multiple pairwise comparisons. *P*-values < 0.05 were considered statistically significant.

Results

Sixty calves were enrolled at T1 and fifty-nine calves in T2; however, only thirty-four calves remained at T3. One calf was removed from the study between T1 and T2 due to severe lameness. Morbidity and mortality of additional calves was not observed during the study period. Reduced calf numbers at T3 were the result of inability to correctly identify calves at T3 due to loss of their identification numbers (n = 20) and leasing of 5 study calves to another producer. There were no significant differences between heifer and bull calves; however, Angus calves were over-represented in the study population with respect to Simmental and Hereford calves (Tables 1 and 2).

Model-adjusted log-transformed means for nasal IgA titers were similar among all treatment groups at each sampling point ($p > 0.1$; Table 3). There was a significant interaction between vaccine protocol and time of sampling with $p = 0.01$ (Table 3; Figure 2). The mean log-transformed BRSV IgA nasal secretion titers were lower at birth compared to samples collected at 2-months and 6-months of age in all treatment groups. At 2-months-of-age and at 6-months-of-age, the mean log-transformed BRSV nasal IgA titers was not different among treatment groups (Table 4). Additionally, Angus calves had significantly higher nasal BRSV IgA titers than Simmental and Hereford calves at each time point irrespective of treatment group ($p < 0.01$; Table 1).

The model-adjusted mean-Log_2 serum BRSV Ab titers were similar among calves from all treatment groups at each sampling point ($p = 0.062$). There was no significant difference in mean log_2 serum Ab titers among vaccination group, breed, or sex at any time period (Figure 1; Table 2).

Discussion

Current vaccination strategies to reduce clinical disease associated with BRSV have not consistently reduced clinical signs in young calves (Ellis et al, 2010; Theurer et al, 2015). The presence of colostrum-derived immunity in serum and the upper respiratory tract are amongst important factors that prevent successful vaccination of calves IFOMA. Increased concentrations of BRSV-specific IgG_1 in nasal secretions has been demonstrated in dairy calves 24 hours after ingestion and absorption of colostrum (Ellis et al, 2018 (1)); however, only specific IgA in the upper respiratory tract has been demonstrated to protect against BRSV or BHV-1 infection

(Kimman et al, 1989; Ellis et al, 2013; Hill et al, 2019). Serum neutralizing BRSV antibodies and nasal secretion BRSV-IgA titers were similar among calves from different vaccine protocol groups in this study. This suggests that vaccination of calves with an IN or SC MLV BRSV vaccine under 24-hours-of-age did not result in better locally or systemically induced BRSV humoral immunity at 2-months-of-age compared with no vaccination. It is possible that absorption of maternally derived antibodies and subsequent translocation to the respiratory mucosa occurs rapidly after colostrum ingestion. The highest apparent efficiency of absorption (AEA) of immunoglobulins from colostrum in calves occurs during the first 6-hours-of-life (Stott et al, 1979; Shivley et al, 2018). Rapid absorption and translocation of passive specific BRSV antibodies could have prevented adequate priming of systemic and local BRSV immunity by vaccination under 24-hours-of-age. Similar to this study, results from another study failed to demonstrate significant differences in serum or nasal BRSV antibody titers in beef calve receiving a combination vaccine protocol at 2-months-of-age compared to calves that received a single dose of a SC MLV vaccine at 2-months-of-age (Ellis et al, 2018 (2)).

The levels of BRSV-IgA noted in NO-IN VAC calves at 2-months-of-age may be the result of translocation of maternal antibodies from colostrum; however, IgA is typically secreted at the lowest concentrations of immunoglobulins (Ig) in colostrum and it has been noted to decay at a more rapid rate than other Igs (Larson et al, 1980). This suggests that the BRSV nasal IgA concentrations observed in NO-IN VAC calves at 2-months-of-age could have been the result of other factors. Inadvertent exposure and subsequent response of NO-IN VAC calves to vaccine BRSV antigens shed by intranasally vaccinated calves could explain the comparable titers of BRSV-IgA in nasal secretions observed at T2. This is entirely possible in this study as NO-IN VAC calves were housed in the same pasture as IN-SC VAC and SC-IN VAC calves after birth.

Shedding of BRSV from nasal secretions has been detected up to 28 days post-vaccination with an IN MLV vaccine in calves (Walz et al, 2017). Exposure to field strains of BRSV circulating within the study herd could also have explained the presence of BRSV IgA titers at 2-months-of-age in NO-IN VAC calves. Results from one study suggested that changes in serum BRSV antibody titers and seroconversion of unvaccinated dairy cows could correspond to exposure to BRSV shed by sub clinically infected cows or calves (de Jong et al, 1996); however, this is unlikely because seroconversion or consistent increase in serum or nasal BSRV antibody titers overtime were not detected among treatment groups.

The levels of BRSV nasal IgA observed at T2 in calves vaccinated at birth (IN-SC VAC and SC-IN VAC treatment groups) could be the result of vaccine priming or transfer of maternally derived BRSV antibodies. Unfortunately, the effect of passive transfer of immunity of BRSV antibodies on local humoral responses is uncertain in calves from this study due to lack of evaluation of passive transfer status. The levels of BRSV nasal IgA at T3 observed in NO-IN VAC calves could have been the result of priming of local immunity. It is possible that the lower levels of BRSV nasal IgA in NO-IN VAC calves at T2 allowed for greater replication of vaccine virus and induction of local immune responses reflected in nasal secretions at T3. In contrast, greater concentrations of BRSV nasal IgA in IN-SC VAC and SC-IN VAC calves at T2 could have reduced the effect of booster vaccination and resulted in lower BRSV nasal IgA at T3 in these calves.

In this study, nasal and serum BRSV antibody titers from calves that were vaccinated with a single IN MLV vaccine (NO-IN VAC) at 2-months-of-age were similar to those that received a combination of IN/SC MLV vaccine protocol at birth and 2-months-of-age (IN-SC VAC and SC-IN VAC groups) at every time point. This suggests that vaccination with a MLV

BRSV vaccine via either a IN or SC route at birth followed by a booster at 2-months-of-age with a MLV BRSV vaccine given by the alternate route may not result in BRSV antibody responses that differ from vaccination with an IN MLV BRSV vaccine at 2-months-age alone. It is possible that BRSV nasal IgA responses induced by vaccination are short lived and that sampling timeframes established in this study did not allow for adequate detection. Previous studies have demonstrated a short duration of immunity (~4 months) when intranasal vaccines are administered IFOMA to dairy calves 3-11-days-of-age (Ellis et al, 2010; Ellis et al, 2013). Similar results have been noted in calves vaccinated at either 0 or 35 days of life against BVDV and BHV1 (Hill et al, 2012). It is possible that multiple doses of an IN vaccine are required to prolong the duration of BRSV-IgA in nasal secretions of calves. Although experimental challenge with BRSV to determine the efficacy of the immune response induced by vaccination protocols would have been ideal in our study, previous studies have demonstrated that serum Ab titers prior to challenge are not a good indicator of protection against BRSV (Woolums et al, 2004; Ellis et al, 2010; Ellis et al, 2018 (2)).

Results from this study failed to identify an advantage of using a combination vaccine protocol at birth and at 2-months-of-age vs. single vaccination at 2-months-of-age in induction of serum or nasal BRSV antibody responses at 6-months-of-age. These findings are consistent with previous reports where combination vaccine protocols did not provide significant advantages on protection against clinical disease when compared to single MLV vaccination (Step et al, 2009; Windeyer et al, 2012).

There were several limitations inherent to this study. There was no control group as all calves received at least one dose of a MLV BRSV vaccine. This prevented the evaluation and potential effect of the decay of local and systemic BRSV specific maternal antibodies. The farm-of-origin was undergoing a change in management at the study time so there was a discontinuity in calf care and accuracy of calf records. The loss of calves between T2 and T3 can be attributed to this, as several calves had ear tags that were missing so treatment group could not be ascertained and therefore accurate sampling data could not be obtained. Along with this, colostrum quality and passive transfer status was not determined during the first 48 hours of life preventing evaluation of exact levels of passively acquired BRSV antibody titers in serum and nasal secretion samples.

Footnotes

1. Bovi-Shield Gold FP5 VL5®, Zoetis, Parsippany, New Jersey.
2. Microsoft Excel®, Microsoft Corporation, Albuquerque, New Mexico.
3. Inforce 3®, Zoetis, Parsippany, New Jersey.
4. Express 5®, Boehringer Ingelheim, Duluth, Georgia.
5. Braunamid® White, Aesculap AG and CO KG, Tuttlingen, Germany.
6. 6 ml Z Serum Clot Activator, Vacuette Tube, Greiner Bio-One North America Inc, Monroe, North Carolina.
7. Nalgene® Cryoware Cryogenic Vials, Thermo Fisher Scientific, Rochester, New York.
8. Ultrabac 8®, Zoetis, Parsippany, New Jersey.

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Figures

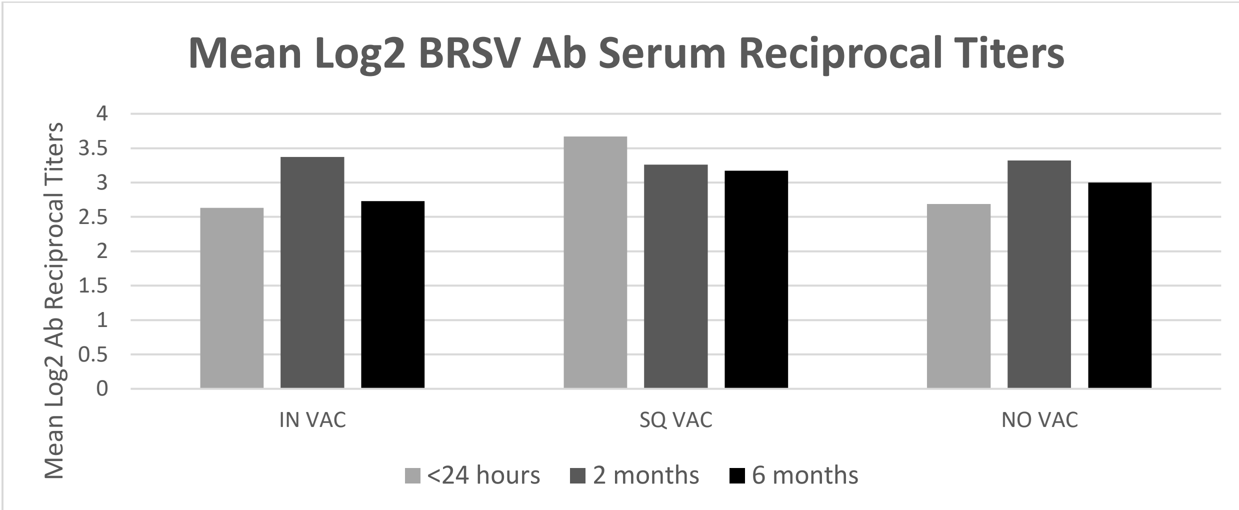

Figure 1 - Mean log2 BRSV Serum Ab Reciprocal Titers

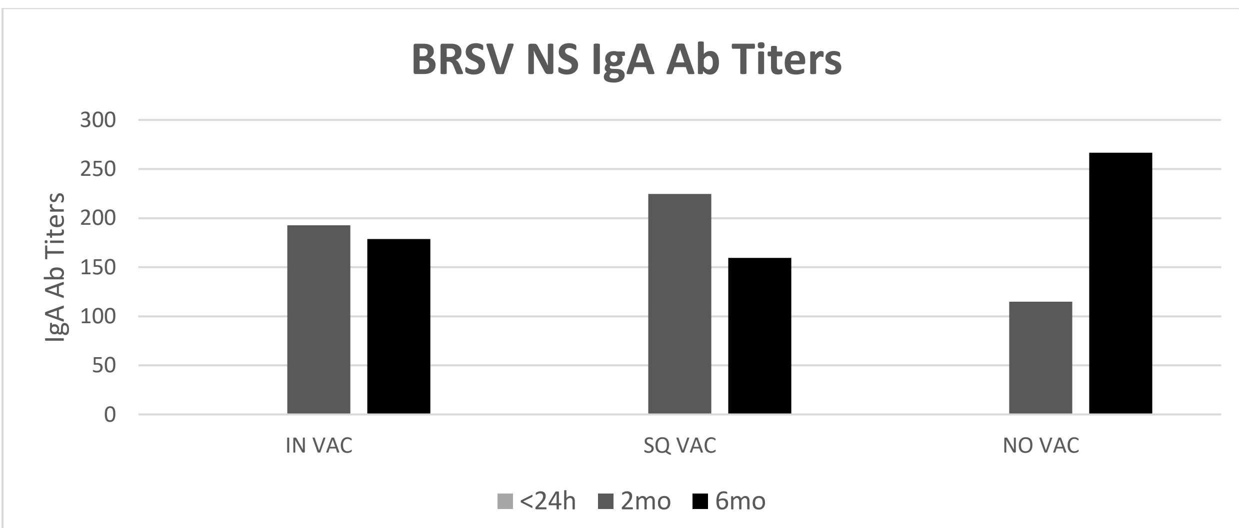

Figure 2 - Mean BRSV NS IgA Ab Titers

Tables

Table 1 - Model-adjusted means, 95% confidence intervals, and P-values for BRSV IgA antibody titers (back-transformed from log10) from univariable linear mixed models. Significant P-values are in bold.

Variable	Mean	95% CI	*P*-value
Vaccine protocol			0.26
IN-SC VAC	17.98	15.08 – 21.45	
SQ-IN VAC	17.86	15.08 – 21.15	
NO-IN VAC	21.52	17.91 – 25.86	
Time of sampling			**< 0.01**
Birth	1.00	0.80 – 1.22	
2-months-of-age	166.42	135.33 – 204.69	
6-months-of-age	194.94	151.84 – 250.26	
Breed			**<0.01**
Angus	23.12	20.60 – 25.94	
Hereford	19.52	15.03 – 25.35	
Simmental	15.85	13.15 – 19.10	
Sex			0.09
Female	17.53	15.30 – 20.08	
Male	20.847	17.97 – 24.18	

Table 2 - Model-adjusted means, 95% confidence intervals, and P-values for BRSV serum antibody titers (in log2) from univariable linear mixed models.

Variable		Mean	95% CI	*P*-value
Vaccine protocol				0.72
	IN-SC VAC	2.94	2.29 – 3.58	
	SQ-IN VAC	3.31	2.66 – 3.96	
	NO-IN VAC	3.11	2.47 – 3.74	
Time of sampling				0.62
	Birth	2.99	2.42 – 3.57	
	2-months-of-age	3.32	2.76 – 3.88	
	6-months-of-age	3.09	2.38 – 3.81	
Breed				0.17
	Angus	2.97	2.50 – 3.43	
	Hereford	3.91	2.99 – 4.83	
	Simmental	2.97	2.22 – 3.71	
Sex				0.65
	Female	3.03	2.52 – 3.55	
	Male	3.20	2.67 – 3.73	

Table 3 - Model-adjusted means, 95% confidence intervals, and P-values for BRSV IgA antibody titers (back transformed from log10) from a multivariable linear mixed model. Significant P-values (P < 0.05) are in bold.**

Variable	Mean	95% CI	*P*-value
Vaccine protocol			0.89
IN-SC VAC	35.55	27.14 – 27.14	
SQ-IN VAC	32.95	32.95 – 39.50	
NO-IN VAC	31.07	31.07 – 37.13	
Time of sampling			**< 0.01**
Birth	1.00	0.81 – 1.22	
2-months-of-age	170.61	139.19 – 209.12	
6-months-of-age	196.61	154.03 – 250.90	
Vaccine protocol x Time of sampling			**0.01**
IN-SC VAC – birth	1.00	0.70 – 1.43	
IN-SC VAC – 2-months-of-age	192.84	134.90 – 275.68	
IN-SC VAC – 6-months-of-age	178.84	116.79 – 273.91	
SQ-IN VAC – birth	1.00	0.69 – 1.44	
SQ-IN VAC – 2-months-of-age	224.49	155.49 – 324.12	
SQ-IN VAC – 6-months-of-age	159.33	105.00 – 241.82	
NO-IN VAC – birth	1.00	0.69 – 1.40	
NO-IN VAC – 2-months-of-age	114.71	82.30 – 159.88	
NO-IN VAC – 6-months-of-age	266.62	174.42 – 407.47	

Table 4 - Model-adjusted means, 95% confidence intervals, and P-values for BRSV IgA antibody titers (back transformed from log10) from a difference of least squared means.

Variable	Mean	95% CI	*P*-value
Vaccine protocol x Time of sampling			
Birth			
IN-SC VAC vs SQ-IN VAC	1.00	0.44-2.27	1.00
IN-SC VAC vs NO-IN VAC	1.02	0.46-2.28	1.00
SQ-IN VAC vs NO-IN VAC	1.02	0.45-2.30	1.00
2-months-of-age			
IN-SC VAC vs SQ-IN VAC	0.86	0.38-1.95	0.99
IN-SC VAC vs NO-IN VAC	1.68	0.77-3.67	0.47
SQ-IN VAC vs NO-IN VAC	1.96	0.89-4.32	0.17
6-months-of-age			
IN-SC VAC vs SQ-IN VAC	1.12	0.43-2.92	1.00
IN-SC VAC vs NO-IN VAC	0.67	0.26-1.76	0.92
SQ-IN VAC vs NO-IN VAC	0.60	0.23-1.55	0.73