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THE ROLE OF PASSIVE IMMUNITY IN BOVINE RESPIRATORY SYNCYTIAL VIRUS INFECTED CALVES

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THE ROLE OF PASSIVE IMMUNITY IN BOVINE RESPIRATORY SYNCYTIAL VIRUS INFECTED CALVES

By

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ABSTRACT

THE ROLE OF PASSIVE IMMUNITY IN BOVINE RESPIRATORY SYNCYTIAL VIRUS INFECTED CALVES

By

Ellen Baker Belknap

The role of passive immunity in bovine respiratory syncytial virus (BRSV) infections in neonatal calves was evaluated. Calves were divided into groups as follows: (I) colostrum-deprived, sham inoculated; (II) colostrum-deprived, BRSV inoculated; and (III) colostrum-fed, BRSV inoculated. Calves were inoculated with a low passage, field isolate of BRSV for four consecutive days by a combined respiratory tract route, and were euthanized 5 days after receiving the last inoculation.

Arterial oxygen tension (P_aO_2) decreased significantly over time in group II calves ($P < 0.01$) and was significantly different between treatment groups ($P < 0.05$). A significant decrease in arterial oxygen saturation was observed in group II over time ($P < 0.01$). Mean values of pneumonic lung volume percentage (determined by computer data digitalization) were significantly greater in group II calves compared to groups I and III. Bovine respiratory syncytial virus antigen was only detected in group II calves by avidin-biotin immunoperoxidase staining. In conclusion, passive immunity derived from colostrum appeared to modify severity of BRSV infections in calves.

This work is dedicated to my mother and father
who taught me the meaning of perseverance, and supported
and encouraged me through the years.
I especially dedicate this thesis to my husband, Jim,
who without his love, understanding, and encouragement
this project would never have been done.

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I. INTRODUCTION

Bovine respiratory syncytial virus (BRSV) has been shown to be an important respiratory tract pathogen of cattle.[1,2,3] Antibody prevalence in the cattle population to BRSV has been reported to range from 65 - 81% in the United States.[4,5] Infections have been reported in adult cattle, feeder age calves, nursing beef calves and dairy calves, although the most common age group affected appears to be calves less than six months of age.[6-9]

Bovine respiratory syncytial virus, a pneumovirus of the family paramyxoviridae, is antigenically similar to the human respiratory syncytial virus (HRSV). Human respiratory syncytial virus is considered to be the major lower respiratory tract pathogen of infants and young children.[10,11] Bovine respiratory syncytial virus and HRSV have been studied in terms of epidemiology, pathogenesis, diagnostic methods, and clinical disease.[5,12,13,15] Controversy exists concerning the role of maternally-derived antibodies in RSV infections in infants and calves, with some reports stating a beneficial effect of maternal antibodies and others reporting a lack of protection or hypothesizing a more severe disease associated with the presence of maternal antibodies.[14,16-23] Laboratory animal models such as cotton rats, ferrets, and mice have been used to try to define the role of passive immunity in experimental RSV infections.[24-29] However, none of these laboratory animals are

natural hosts of RSV and results from these studies have been equivocal in their findings regarding the role of passive immunity in RSV infections. Calves are a more logical model to study the role of passive immunity in RSV infections for several reasons: 1) calves are naturally infected with BRSV, 2) BRSV infections are similar to HRSV with regards to epidemiology, clinical signs, pathologic lesions and pathogenesis, and 3) passive transfer of immunity can be controlled by colostrum feeding.

The purpose of the present study was to determine the difference in response to BRSV infections in calves with and without passive immunity.

II. RESPIRATORY SYNCYTIAL VIRUSES

Human respiratory syncytial virus and BRSV are both members of the pneumovirus genus of the family Paramyxoviridae. Both viruses are negative-stranded enveloped RNA viruses which code for at least 10 gene products.[11] Human respiratory syncytial virus was initially isolated from a chimpanzee, although not a natural host for HRSV, and then one year later it was isolated from infants.[30] Approximately 10 years later, in 1970, BRSV was isolated from cattle.[31] Sheep and goat respiratory syncytial viruses have been identified, but have not been studied in depth.[1]

Although antigenically related, BRSV and HRSV are two distinct viruses.[5] Whereas HRSV has been shown to infect cattle experimentally, BRSV has not been isolated from humans, although this has not been studied in detail.[32] However, the

two viruses do have many similarities including classification, epidemiology, clinical signs, pathologic lesions, and possibly pathogenetic mechanisms.[11,33,34] Two subgroups, A and B, have been designated for HRSV isolates which are based on two different patterns of reactivity with monoclonal antibodies directed against the four major structural proteins of HRSV.[35] It remains to be determined if subgroups of BRSV exist.

III. HUMAN RESPIRATORY SYNCYTIAL VIRUS

Human respiratory syncytial virus is the most important respiratory virus affecting young children, with approximately fifty percent of infants becoming infected during their first year of life, and the remainder during their second year of life.[15,33,34,36] A seroepidemiologic study reported 50% of children aged 6 - 48 months in an open population to have detectable serum antibodies to HRSV.[37] Reinfection occurs commonly in older children and adults, with the disease being an upper respiratory tract disease which is less severe than the lower respiratory tract disease seen in young children.[10]

Antigenic variation is not thought to be the mechanism for reinfection, and the fact that immunity to HRSV is only short-lived may be the most plausible explanation for reinfection.[10,34,38] Transmission of the virus is thought to occur most commonly through respiratory secretions and direct contamination, but not by aerosolization of small particles.[10] Epidemics of HRSV are reported regularly

around the world with a seasonal distribution for late fall, winter, and early spring.[10,39]

Coughing, rhinitis, dyspnea, wheezing, and fever are characteristic signs of the disease in infants and young children. Rales and rhonchi may be heard on thoracic auscultation.[11] Recurrent wheezing or asthma may follow acute HRSV infections in some children.[34]

RSV is probably an important cause of death in infants less than one year of age, and even higher mortality rates occur in infants with RSV infections complicated by other illnesses.[34] Pathologic lesions include necrosis of the tracheobronchiolar epithelium, peribronchiolar lymphocytic infiltration, interstitial pneumonitis, and focal areas of atelectasis and emphysema due to bronchiolar obstruction.[11]

IV. BOVINE RESPIRATORY SYNCYTIAL VIRUS

Bovine respiratory syncytial virus was first isolated from cattle in 1970 and is currently recognized as a common cause of respiratory tract disease in cattle.[1,2,3,9,31] The disease is usually associated with a high morbidity and low mortality rate, although case fatality rates can reach 20 per cent.[1,40] Mortality may be related to the occurrence of secondary bacterial infections of the lower respiratory tract.[2] BRSV usually infects younger cattle and is prevalent in the fall and winter months.[42]

Clinical signs of the disease include a sudden onset of respiratory signs characterized by serous naso-ocular discharge, coughing, and pyrexia (39.4 - 42.2°C).[43,44] Other respiratory signs including tachypnea, and, occasionally, an abdominal component to respirations are observed.[2,8,43] Subcutaneous emphysema may occur due to rupture of the lung secondarily to airway obstruction, hypoxia, and forced respirations with subsequent migration of air to the subcutaneous tissue through the mediastinum.[1,2] A secondary bacterial infection of the lungs is frequently associated with BRSV infections.[2,41]

Gross postmortem examination of the respiratory tract from infected cattle reveals lungs that generally do not collapse due to interstitial emphysema. Additional findings include emphysematous bullae, subpleural emphysema, and interstitial edema and emphysema.[1] Consolidation may be present in the cranioventral portion of the lungs.[45] Histopathologic lesions include bronchitis and bronchiolitis.[45] Syncytial giant cell formation in bronchiolar and alveolar lumina are frequently observed.[8] Hyaline membrane formation is sometimes present along with thickening of alveolar walls due to proliferation of type II alveolar epithelial cells, edema and cellular infiltrate.[45]

V. ROLE OF PASSIVE IMMUNITY IN RSV INFECTIONS

A. Human Respiratory Syncytial Virus

Temporary protection against infection may be accomplished by passive immunization, which is the transfer of antibodies from one individual to another of

the same or different species.[46] Cell-mediated immunity can also be transferred to the neonate via colostrum which contains B and T lymphocytes.[46] Humans have a hemochorial type of placentation which allows for approximately 90% of immunoglobulins to be transferred in utero and only 10% via colostrum.[46]

Although very little maternal immunoglobin is transmitted through colostrum in humans, breast feeding of infants and its relation to HRSV infection has been a debated subject since the 1970's. Several studies found that the incidence of HRSV was less in those infants that were breast fed as opposed to the formula-fed controls.[15,47,48] Other studies, which took into account social and familial factors, failed to show a significant correlation between breast feeding and incidence or severity of lower respiratory disease associated with HRSV infection.[48,50,51] A study in 1982 showed that although there were no statistically significant differences in rates or distributions of infections between breast fed and formula fed infants, there were trends toward decreased episodes of respiratory infections (pneumonia and bronchiolitis) during the first 6 months in breast-fed infants as compared to bottle-fed infants.[52]

Human respiratory syncytial virus-specific IgA, IgG and IgM antibodies have been identified in colostrum.[47] In these colostrum samples the IgA titer was higher and correlated more closely with the titer of HRSV neutralizing activity in the colostrum than did the IgG titers.[47,53] Levels of colostral antibody decline after the first week of lactation, making it difficult to explain protection afforded by colostral transfer of immunoglobins as the sole factor responsible for positive effects of

breast feeding.[34,53] However, HRSV-specific IgA has been found to reappear in milk of lactating mothers when seasonal epidemics of HRSV occur, suggesting a natural booster effect and a possible contribution of sensitized IgA-secreting lymphocytes of respiratory tract origin to RSV-specific immunologic reactivity in lactational products.[54]

Reactive cells to HRSV, as determined by a transformation assay and presumed to be of mucosal lymphoid origin, were detected in one-third of colostral samples during a HRSV epidemic.[55] The reactivity of the cells did not correlate with levels of HRSV-specific IgA and IgG in colostrum and maternal serum, indicating that cellular and antibody responses may function independently. This HRSV-specific cellular reactivity could be responsible for the protection against RSV infection associated with breast-feeding.[34,55]

For the last 20 years, the role of maternally derived antibody in the pathogenesis of HRSV infection has been controversial. Initially, in the late 1960's, it was observed that the highest incidence of HRSV and severe disease manifestations was in infants less than 6 months of age, a time when maternal antibodies were present. This led to the hypothesis that the presence of maternal antibody in the infant could enhance disease severity. It was then speculated that an immunopathologic phenomenon may be involved in the production of disease in this age group.[16,56] Currently, immunopathologic mechanisms, such as type III hypersensitivity, are not thought to contribute to the disease, since positive correlation has not been found between the acute levels of serum antibody to

HRSV and the severity of disease.[14,21,57] Also, infants less than 3 weeks of age, who have the highest levels of maternal antibody specific for HRSV, appear to be spared from HRSV infections.[14] In addition, the presence of antibody does not appear to be an essential part of HRSV infections as disease occurs in infants who have no measurable HRSV-specific antibodies.[57]

A suppressive role of maternal antibodies was demonstrated in a 1973 epidemiologic study which found infants with high levels of acute phase antibody to HRSV had undetectable or only low levels of complement fixation (CF) antibody response during convalescence, suggesting that maternal antibodies were responsible for suppressing the humoral immune response of infants.[14] In contrast, some 5 - 7 month old children infected with HRSV had very low or undetectable acute levels of HRSV antibody in their acute phase serum, questioning the contribution of antibody to the disease response occurring in HRSV infections in infancy.[14] Another study did not show a correlation between severity of illness in relationship to preexisting antibody titers in umbilical cord sera or acute systemic sera as measured by complement fixation (CF).[57] The severity of HRSV pneumonia in a later study was reported to be inversely related to the level of maternally-derived neutralizing antibody, although this was not true with HRSV-caused bronchiolitis in infants.[21] Maternally-derived antibody did not worsen the severity of either of the illnesses as previously hypothesized. In a 1981 study, mean titers of HRSV-specific IgG antibody in maternal serum were significantly higher ($P < 0.01$) for infants who did not become infected with HRSV than those who did become

infected.[20] Another study that same year demonstrated infants born with higher HRSV antibody levels became infected later in life, while infants with moderate antibody titers had less severe illness than infants with little or no antibody titers.[17] It was also noted that infants with maternal antibody titers of 1:16 or more (determined by a serum neutralizing test) were unlikely to develop severe disease.[17] Thus, it appears that children with either high or negligible levels of maternal HRSV-specific antibodies became infected with HRSV, although some studies have found that higher levels of maternal antibodies specific for HRSV may have some protective effect against HRSV infections.[14,17,20] It also is demonstrated that higher levels of maternal antibodies specific for HRSV may suppress the infant's immune response following infection with HRSV.[14]

Support for the hypothesis that HRSV-induced disease may be due to an antibody-antigen reaction stemmed from two vaccine trials in the late 1960's which found that high levels of neutralizing antibody against HRSV produced by vaccination did not offer protection when the children were naturally infected with HRSV.[56,58] Vaccinates receiving a formalin-inactivated HRSV vaccine were more severely affected when infected with HRSV than the controls who received the formalin-inactivated parainfluenza virus vaccine. It was thought that the increased severity of the illnesses in the vaccinates was due to an interaction between the viral antigen and serum antibody.[56] Theories for this vaccine induced enhancement of disease response include: 1) altered response to inactivated virus, 2) serum

antibody without secretory nasal antibody was immunopathogenic, and, 3) delayed hypersensitivity due to the vaccine.[56,58]

Recent research has elucidated the observed phenomena of vaccine enhancement of disease response. The fusion (F) glycoprotein of HRSV stimulates production of both a neutralizing and a fusion-inhibiting antibody.[59] Evaluation of stored sera samples from the vaccinates in the formalin-inactivated HRSV vaccine trial identified antibodies against the F protein that were deficient in neutralizing and fusion-inhibiting activity.[60] This meant the formalin-inactivated vaccine induced an antibody response directed towards the non-protective epitopes on the F glycoprotein, resulting in high levels of ineffective serum antibody against HRSV.[61] Although antibody-antigen complexes could have formed, the exacerbation of HRSV observed in the vaccine trial probably was not directly related to increased levels of serum antibody against HRSV, but more likely due to a lack of the correct antibody response to HRSV. Thus, it appears that vaccine enhancement of disease response to HRSV most likely involves a different mechanism and does not necessarily relate to pathogenetic mechanisms involved in the naturally occurring disease.

Laboratory animals have been used to study the role of passive immunity of HRSV. Ferrets do not have placental transfer of antibodies whereas cotton rats have both transfer across the placenta and through colostrum.[24] In ferrets, gestational infection of mothers offered protection through colostrum to infant ferrets challenged at 3 days of age with HRSV, and the level of protection was

directly related to the level of maternal neutralizing antibody in the mother.[25] In contrast, oral or intraperitoneal administration of serum with high neutralizing titers did not confer protection against viral replication in infant ferrets.[25]

The passive administration of serum antibody to cotton rats suppressed the humoral immune response when followed by intramuscular vaccination with live HRSV, but cotton rats given intranasal inoculation with live HRSV were protected from a subsequent challenge with HRSV.[28] Maternally-derived passive antibody to HRSV in the infant cotton rat was found to be only of transient duration (detectable for 1 week in the nasal tissue and 4 weeks in the lungs).[24] This passively-derived immunity in infant cotton rats is represented by serum neutralizing antibody level correlated with protection following challenge, but there were exceptions, suggesting that other factors besides neutralizing antibody may be responsible for passive immunity to HRSV.[24] A subsequent study by the same group determined that passive serum neutralizing antibody titers of greater than 1:100 suppressed viral replication in the respiratory tracts of cotton rats serum neutralizing antibody titers of greater than 1:380 conferred almost complete resistance in the lungs to HRSV challenge.[27] Using these data, a dose relationship was determined between HRSV antibody levels and HRSV protection in the respiratory tract. Further work on passive immunity in cotton rats demonstrated that both transplacental and lactogenic antibodies for HRSV were able to reduce viral replication in respiratory tracts of infant cotton rats.[29] However, the

protection was only transient. In 2-month-old animals, previous passive acquisition of maternal antibodies resulted in no reduction of viral replication.[29]

Monoclonal antibodies (MAB) with specificity against the large (G) and F envelope glycoproteins of HRSV have been used in studies in mice and cotton rats.[26,62,63] After passive administration of MAB to HRSV by intraperitoneal injection to cotton rats, lower mean viral titers were found in lung tissue after virus challenge, and a decrease in viral replication was observed in nasal turbinates as compared to control cotton rats.[26] These data were confirmed in mice where passive immunization with MAB to HRSV resulted in significant reductions in virus titer in the lungs after nasal inoculation with live HRSV.[62] Two bovine MABs with specificity against the F glycoprotein were demonstrated to inhibit virus-induced fusion, lyse HRSV-infected cells and protect mice when inoculated with HRSV.[63]

A possible protective role of antibodies to glycoproteins of HRSV has been identified. The four structural proteins used to differentiate HRSV isolates between subgroups A and B are the F and G glycoproteins, nucleoprotein (NP), and matrix protein (M).[35] After HRSV infection in children, antibody responses to the F glycoproteins of either strain were similar as opposed to responses to the G glycoproteins of subgroups A and B, which were significantly lower in subgroup A ($P < 0.005$).[64] Over a number of epidemics, a predominant subgroup strain (A or B) was observed, suggesting a degree of subgroup specific immunity, however a change in the subgroup was observed over a long period of time.[65] Although no differences in the clinical illnesses were observed between the subgroup A and B

infections, subgroup A occurred 3 times more often than subgroup B in initial infections.[66] With reinfections, this ratio was not demonstrated and there was no evidence of cross-immunity between the two strains, although some protection against a second infection was afforded by the homologous strain.[35]

The latest data suggest that F and G glycoprotein-specific antibody responses to HRSV infections are affected by age and maternal antibody level against HRSV. Age primarily affects the response to the F glycoprotein with older children having a greater response to the F glycoprotein, and preexisting maternal antibody titer predominantly affects the response to the G glycoprotein.[67] High levels of maternal antibody were observed to suppress antibody response to the G glycoprotein in children infected with HRSV up to approximately 8 months of age.[67] This suggests that both immaturity of the immune system and antibody-mediated suppression of the humoral immune system affect the immune response of the infant infected with HRSV.

Numerous experimental studies to determine the role of passive immunity in RSV infections have been performed with laboratory animal models. The results of these studies have been equivocal. Epidemiologic studies of infants infected with HRSV suggest a possible protective role of passive immunity, but the precise role has not been determined.

B. Bovine Respiratory Syncytial Virus

The epitheliochorial type of placentation found in cattle prevents the transfer of immunoglobulins to the fetus in utero. Thus, calves are born agammaglobulinemic and are totally dependent upon intestinal absorption of immunoglobulins derived through ingestion of colostrum during the first forty-eight hours of life.[46] In bovine colostrum, the immunoglobulins comprise greater than 95% of the total whey protein and IgG_1 accounts for approximately 80% of this amount.[68] IgG_2 (2.87 mg/ml) is much lower in concentration in bovine colostrum, although in calf serum IgG_1 and IgG_2 are in nearly equal amounts.[68] IgA (5.36 mg/ml) and IgM (6.77 mg/ml) mean levels are similar in colostrum, but lower than IgG_1 (46.4 mg/ml).[68] Maternal antibodies to BRSV are restricted to the IgG_1 isotype in serum.[69]

There are few reports of the role of maternally-derived antibody in association with natural infections of BRSV, and studies in the 1970's and 80's have demonstrated that preexisting antibody titers to BRSV are not protective against BRSV infection.[22,70,71,72] In one study, maternal antibodies failed to protect newborn calves from BRSV infections.[70] In other natural outbreaks of BRSV, on the first day of illness calves were found to have moderate to high acute levels of BRSV-specific IgG_1 which were most likely maternally derived. These calves developed severe signs of respiratory disease.[22] A later epidemiological study found that BRSV was infrequently found in calves less than two weeks of age, regardless of antibody levels, while the most severe BRSV associated disease occurs in calves

one to three months of age, and the severity of these infections was inversely related to the level of maternal antibody.[23] This was the first study to suggest that maternal antibodies have a protective role in BRSV infections.

It has been demonstrated that calves experimentally infected with BRSV in the presence of BRSV-specific maternal antibodies may develop disease.[5,73] It does not appear that the presence of maternal antibody to BRSV exacerbates the severity of the disease.[18,32]

A recent study on passive immunity in calves found that colostrum-fed and colostrum-deprived calves shed virus in approximately equal amounts and for an equal amount of time after experimental infection with BRSV, and both groups developed mild signs of disease.[22] Maternally derived antibodies have been demonstrated to suppress both local and systemic antibody responses to BRSV infection, with IgM levels being the least suppressed.[19,22] The degree of antibody suppression was directly related to the level of BRSV-specific serum IgG_1 preinoculation, with higher levels coinciding with a greater suppression of antibody responses post-inoculation.[22] Similarly to infants, a large proportion of calves infected with BRSV are 1 - 3 months of age, a time when maternal antibody levels to BRSV are present and could interfere with antibody production following vaccination for BRSV.[70]

Moderate levels of maternally-derived antibodies to BRSV, particularly those directed towards the F and N proteins, were found to be nonprotective in a group of 2 - 3 week old calves infected with BRSV.[19]

Thus, in both calves and infants the role of passive immunity has not been clearly defined. Three different roles have been suggested for maternal antibodies have been: 1) exacerbation of the severity of HRSV infections, 2) lack of protection against RSV infections, and 3) modification of the severity of RSV infections. The first hypothesis has been disproven, while a recent study supports the third possibility, that maternal antibodies specific to BRSV may have a protective effect on the disease.

VI. OBJECTIVES

The specific aim of this study was to determine the difference in response to BRSV infection in calves with passive immunity (colostrum fed) and calves without passive immunity (colostrum deprived). Parameters measured and statistically compared between and within groups included body temperature, respiratory rate, pulse rate, arterial blood gases, and quantitative volumetric measurement of pneumonic lung. Descriptive histopathologic evaluation of lungs was performed and the presence and distribution of BRSV antigen in the lung was determined by immunoperoxidase staining.

VII. MATERIALS AND METHODS

A. Calves

Holstein bull calves were obtained from a local dairy. All calvings were attended and full-term calves were delivered onto plastic sheeting and taken

immediately to isolation facilities. Each calf was housed individually in an elevated crate in a separate room from other calves. Injections of 500,000 IU vitamin A[a], 75,000 IU vitamin D[a], 200 IU of d-alpha tocopherol acetate[b], and 3 mg of selenium[b] were given intramuscularly at birth. A single dose of Genecol 99[c] (a monoclonal antibody to K-99 antigen of E. coli) was given orally to each calf at birth. The calves were administered the antibiotic ceftiofur sodium[d] at 0.5 mg/lb once daily for the duration of the study by intramuscular injection. Calves were fed a non-medicated milk replacer[e] at 10% of body weight divided into two equal feedings per day for the duration of the experiment. Colostrum-fed calves were fed colostrum for the first four feedings and then maintained on milk replacer.

B. Experimental Design

Three groups of calves were used in the study:

Group I - Five colostrum-deprived calves sham inoculated with non-infected tissue culture fluids,

Group II - Six colostrum-deprived calves inoculated with BRSV,

Group III- Six colostrum-fed calves inoculated with BRSV.

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[a] Vitamin A & D Injectable Solution, Pfizer, Inc., New York, NY.

[b] Bo-Se, Schering Corp., Kenilworth, NJ.

[c] Genecol 99, Schering Corp., Omaha, NE.

[d] Naxcel, The Upjohn Co., Kalamazoo, MI.

[e] Fresh Start, Vita Plus Corp., Madison, WI.

All calves were maintained for twelve days and then euthanized for post-mortem examination, except one calf in group II which was euthanized on day 11.

C. Colostrum

Holstein dairy cows, seropositive to BRSV, were vaccinated with a BRSV vaccine[f] twice at three week intervals prior to calving. Colostrum from these cows was collected at the first milking after calving, pooled, and stored at -20°C. Each calf in group III received two quarts of colostrum at birth and again at twelve hour intervals for a total of four feedings. After the four feedings of colostrum, group III calves were then maintained on milk replacer as previously described.

D. BRSV Inoculum and Inoculation of Calves

1. Cell Culture

The virus was propagated in bovine turbinate cells[g] maintained in Eagle's minimal essential medium[h] (EMEM) to which 10% fetal bovine serum[i], gentamicin sulfate (25 μg/ml), sodium penicillin G (50 μg/ml), streptomycin sulfate (50 μg/ml), neomycin sulfate (100 μg/ml), and amphotericin B (2.5 μg/ml) were added.

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[f] BRSV™, Norden Laboratories, Inc., Lincoln, NE.

[g] National Veterinary Service Laboratory, Ames, IA.

[h] KC Biological, Lenexa, KA.

[i] HyClone Laboratories, Logan, UT.

Cultures were incubated at 37°C in 5% CO_2. Binary ethyleneimine was used to inactivate adventitial viruses in the bovine fetal serum as previously described.[74] Bovine turbinate cell cultures were shown to be free of bovine viral diarrhea virus (BVDV) by direct immunofluorescence testing.

2. Isolate

A field isolate of BRSV obtained from an outbreak of respiratory tract disease in calves in Minnesota was used in this study.[9]

3. Propagation of Virus

Prior to infecting cell cultures with BRSV, the EMEM with fetal bovine serum was replaced by EMEM containing 10% fetal horse serum and antibiotics as previously described. Tissue culture flasks were inoculated with fourth passage stocks of BRSV when cell monolayers had reached 90% confluency. Virus was harvested when 20 - 25% of the cell monolayer displayed virus-induced cytopathic effects, usually at 72 - 96 hours. The media was decanted and replaced with EMEM containing 44% sucrose and 20% horse serum. The cell cultures were frozen at -70°C and thawed for five cycles to promote cell rupture and release of virus into the sucrose media. The decanted tissue culture medium was used as the intranasal inoculum (Inoculum A) and the EMEM with sucrose containing the disrupted cell monolayer was used as an intratracheal inoculum (Inoculum B). Inoculum for the control group (group I) was prepared in the same manner using non-infected monolayers of bovine turbinate cells.

4. Bacterial and Viral Screening of BRSV Inoculum

The virus inoculum was cultured and determined to be free of aerobic bacteria, mycoplasma, and ureaplasma following standard procedures. BRSV inoculum was also tested for the presence of noncytopathic BVDV by inoculation of a colostrum-deprived calf which was seronegative to BVDV. The calf was given a subcutaneous inoculation of one ml of fourth-passage BRSV for three days and seroconversion to BVDV was not observed over the following three weeks. To demonstrate the calf was immunocompetent to BVDV, the calf was injected with a vaccine containing modified-live BVDV[j] three weeks after the subcutaneous inoculation of BRSV. Seroconversion was demonstrated following BVDV vaccination.

5. Titration of Virus

The intranasal inoculum (Inoculum A) contained between $10^{2.72}$ and $10^{3.8}$ $TCID_{50}$ of BRSV/ml. The intratracheal inoculum (Inoculum B) contained between $10^{4.8}$ and $10^{5.9}$ tissue culture infective doses ($TCID_{50}$) of BRSV/ml. The viral titers of the two inocula were determined according to the method of Carbrey[75], and the 50% endpoint of the virus titer was calculated by the method of Karber[76].

6. Inoculation of Calves

Calves were inoculated with BRSV according to the protocol described by Bryson[77] as modified by Ciszewski[78] starting on experimental day 3 (day 3 of life)

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[j] Resbo 3, Norden Laboratories, Inc., Lincoln, NE.

and were inoculated for 4 consecutive days. Day 3 was chosen as the starting date to allow for maximum intestinal absorption of colostral immunoglobulins in group III (colostrum-fed calves). Groups II and III calves received 10 ml of inoculum A intranasally and 10 ml of inoculum B intratracheally each morning. Each calf received 10 ml of inoculum A intranasally each evening. Group I calves were inoculated similarly except that non-infected tissue culture fluids were used. The non-infected tissue culture fluids were prepared in the same manner as previously described for the BRSV inocula. Prior to administration of the inoculum, calves were made to hyperventilate by forced rebreathing into a small plastic bag.

E. Monitoring and Sample Collection

Calves were observed twice daily. Respiratory rate, heart rate, and body temperature were recorded. Serum was collected prior to inoculation on day 3 and immunoglobin G levels were measured by radial immunodiffusion by the Clinical Pathology Laboratory at Michigan State University.[79] Serum was collected from calves at birth and at days 3, 6, 9, and 12 to measure antibody titers to BRSV, BVDV, IBRV, and PI_3 virus. Sera were stored at -20°C and serotesting was simultaneously performed on all samples after the experiment had concluded. Nasal swab[k] specimens were taken from each nostril on day 8 (2 days after the last inoculation) for virus isolation. The external nares were cleaned of nasal discharge

[k] Dacron Swab, American Scientific Products, McGraw Park, IL.

with 4 x 4 gauze sponges and swabs were then inserted their full length, gently rotated, removed and placed into 2 ml of transport media which consisted of EMEM with 10% horse serum and antibiotics as previously described.

Arterial blood for blood gas determination was taken on days 3, 5, 7, 9, and 11. Heparinized arterial blood samples were taken via arteriopuncture of the thoracic aorta with an 18G 4" needle as previously described.[80] Arterial blood gas tensions were measured using a blood gas analyzer[l] and values were corrected for rectal temperature.[81]

F. Serologic Testing

Serology for BRSV was performed by a microtiter serum neutralization procedure as previously described.[78] Serology for IBRV, PI_3, and BVDV was performed by the Animal Health Diagnostic Laboratory at Michigan State University.[82] Serology for IBRV and BVDV was performed by virus neutralization and by hemagglutinization inhibition for PI_3.

G. Virus Isolation

1. BRSV

Nasal swab specimens were inoculated onto bovine turbinate cells grown in Costar[m] 24-well tissue culture plates. Adsorption was allowed to occur for 1 hour

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[l] Moder ALB-3, Radiometer, Copenhagen, Denmark.

[m] Costar, Cambridge, MA.

at room temperature, and the cell layer was then rinsed three times with Hanks balanced salt solution (HBSS), and then 10% horse serum and antibiotics at levels previously described were added. The plates were incubated at 37°C in a 5% CO_2 humidified atmosphere and observed daily for virus-induced cytopathic effects. Confirmation of BRSV was by direct immunofluorescence staining.[n] After 7 days, coverslips were stained with a direct fluorescent antibody conjugate against BVDV[o] to test for the presence of noncytopathic BVDV.

2. Other Respiratory Viruses

Routine virus isolation on nasal swab specimens was performed by the Animal Health Diagnostic Laboratory at Michigan State University. The samples were inoculated onto bovine turbinate cells grown on coverslips in Leighton tubes. The cell monolayer was examined daily for viral cytopathic effect following inoculation. If cytopathic effect was observed, the isolated virus was identified by direct immunofluorescence. At the end of 7 days if no cytopathic effect was observed, coverslips were stained with a direct fluorescent antibody conjugate against BVDV[p] to test for the presence of noncytopathic BVDV.

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[n] conjugated anti BRSV antibody, Dr. Merwin Frey, University of Nebraska, Lincoln, NE.

[o] National Animal Disease Laboratory, Ames, IA.

[p] National Animal Disease Laboratory, Ames, IA.

H. Postmortem Sampling

Calves were euthanized by barbiturate[q] overdose on day 12. Just prior to euthanasia, each calf was administered 50 IU/kg heparin[r] intravenously. The respiratory tract was removed, photographed, and gross lesions were recorded. Fixation of the lungs was accomplished by cannulation of the pulmonary artery and trachea followed by simultaneous perfusion of 10% buffered formalin through the vasculature at 30 cm H_2O and through the trachea at 10 cm H_2O. The system was closed as all vessels were clamped.

Each lung lobe was cut into 1.3 cm thick sections in the dorsoventral plane. The caudal lung lobes were sectioned using a meat slicer[a] and the remaining lobes were sectioned with tissue slicer blades[b] on a gridded board. The total and pneumonic lung lobe areas of each section were traced onto acetate sheets. Using a puck and a digitizing tablet[c] connected to a Zenith 386 computer, the tracings were entered into a program[d] designed to create 3-dimensional displays and to determine volumes of serially sectioned data. The program was used to determine the total and pneumonic lung lobe volume.

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[q] Fatal-Plus, Vortech Pharmaceuticals, Dearborn, MI.

[r] Heparin sodium, LymphoMed, Inc., Melrose Park, IL.

[a] Omas USA, Inc., Dearborn Heights, MI.

[b] Thomas® Tissue Slicer Blades, Thomas Scientific, Swedesboro, NJ.

[c] Jandel Scientific, Corte Madera, CA.

[d] PC3D™, Jandel Scientific, Corte Madera, CA.

Sections from pneumonic and normal appearing areas from each lung lobe were taken for histopathologic examination and processed routinely. Tissue blocks of formalin-fixed paraffin embedded lung were submitted from each calf for immunoperoxidase staining for BRSV antigen as previously described.[83,84] The antibody employed in the immunoperoxidase procedure was a polyclonal antibody to HRSV[e].

I. Statistical Analysis

A one-way analysis of variance (ANOVA) for repeated measures was used to evaluate daily collected data for each group over time (temperature, heart rate, respiratory rate), and data for arterial oxygen tension (P_aO_2), arterial carbon dioxide tension (P_aO_2), % oxygen saturation, and hemoglobin levels.[85] The Tukey omega procedure was used to evaluate the differences between the means when the F value was significant ($P < 0.05$). A split-plot ANOVA was used to evaluate between group differences of the data previously mentioned and the % pneumonic lung volume. Bonferroni's two tailed t test was used to evaluate significant differences between groups ($P < 0.05$) for all data except % pneumonic lung volume. The test for Least Significant Differences was used to evaluate significant differences between groups for % pneumonic lung volume.

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[e] Dako, Dimension Laboratories Inc., Mississauga, Ontario, Canada.

VII. RESULTS

A. Body Temperature, Heart Rate, Respiratory Rate

All groups had a significant increase in body temperature over time (Figure 1). Group II had significantly higher temperatures ($P < 0.05$) on days 8, 9, and 10 than group I. Group III had a significantly higher temperature ($P < 0.05$) on day 9 than group I, and group II had a significantly higher temperature ($P < 0.05$) on day 10 than group III. The highest temperature was observed in group II calves on day 10 (39.7°C). Although there was a significant increase over time ($P < 0.01$), temperatures in group I calves never exceeded values within the normal accepted range of body temperature for calves (37.8 - 39.2°C).[86]

A significant increase in respiratory rate was present in group II calves, and a significant decrease in respiratory rate in group I calves (Figure 2). There was a significant difference in respiratory rate between groups ($P < 0.05$). These differences were found between groups I and II, and groups II and III on days 9, 10, 11, and 12, and in both instances group II calves had the higher respiratory rates.

There was no significant change in heart rate over time in group I or III calves (Figure 3). Group II calves displayed a significant increase in heart rates over time ($P < 0.05$). There was no significant difference between groups over time, although a significant treatment and time interaction was seen between all groups ($P < 0.05$).

B. Blood Gas Tensions

A significant decrease in P_aO_2 was observed in group II calves over time ($P < 0.01$) (Figure 4). No significant decrease in P_aO_2 was present in group I or III calves over time. There was a significant difference between treatment groups ($P < 0.05$) with group II calves having significantly lower P_aO_2 than groups I and III. These significant differences in P_aO_2 were observed between groups I and II on days 9 and 11, and between group II and III on days 7 and 11.

A significant decrease in % O_2 saturation was observed only in group II calves ($P < 0.01$) (Figure 5), but there was no significant difference between groups. There were no significant differences within or between groups in P_aCO_2 (Figure 6). Significant increases in mean hemoglobin levels were observed over time in all groups of calves (Figure 7) and group III had significantly higher hemoglobin levels ($P < 0.05$) on days 3, 5, 7, 9, and 11 than group I.

C. IgG Determination

Serum IgG levels ranged between 0 to 397 mg/dl for calves in groups I and II (Table 1). Group III calves serum IgG levels ranged between 914 to 2082 mg/dl.

D. Serology

None of the calves possessed serum antibodies to BRSV at birth. All group I calves (Table 2) remained seronegative throughout the experiment while group

II calves displayed seroconversion to BRSV (four-fold rise in titer or a change from undetectable to detectable levels of antibody) by day 12 (six days following the last inoculation with BRSV). After feeding colostrum to group III calves, specific antibody levels to BRSV increased from nondetectable to a geometric mean antibody titer of 1:256 by experimental day 3 (Figure 8) and remained stable or decreased over time.

Group I and II calves did not seroconvert to BVDV, IBRV, and PI_3 virus. Following ingestion of colostrum, group III calves displayed a rise in antibody titer to all three viruses.

Table 1. Immunoglobin G levels (mg/dl) of calves. Serum was taken on day 3 prior to inoculation with BRSV. Groups I and II were colostrum deprived and group III was colostrum fed.

Group I		Group II		Group III	
Calf #	IgG (mg/dl)	Calf #	IgG (mg/dl)	Calf #	IgG (mg/dl)
1	0	11	< 33	21	1604
2	43	12	< 33	22	1372
3	397	13	0	23	2082
5	< 33	15	< 33	24	914
6	< 33	16	< 33	25	955
		17	< 33	26	1360

Table 2. Reciprocal antibody titers to BRSV in groups I, II, and III calves. Group I calves were colostrum deprived and inoculated with tissue culture media. Group II calves were colostrum deprived and inoculated with BRSV. Group III calves were colostrum fed and inoculated with BRSV. Calves were inoculated on days 3 - 6.

Calf Number	Day 1	Day 3	Day 6	Day 9	Day 12
Group I					
1	N	N	N	N	N
2	N	N	N	N	N
3	N	N	N	N	N
5	N	N	N	N	N
6	N	N	N	N	N
Group II					
11	N	N	N	2	4
12	N	N	2	2	4
13	N	N	2	2	8
15	N	N	2	2	8
16	N	N	N	2	*
17	N	N	N	2	4
Group III					
21	N	256	256	256	256
22	N	256	256	256	128
23	N	256	256	256	256
24	N	256	128	128	128
25	N	256	128	128	64
26	N	256	256	128	128

N = BRSV titer < 2

* Calf # 16 was euthanized on day 11

E. Virus Isolation

Bovine respiratory syncytial virus was not isolated from nasal swab specimens collected from calves in group I, but was isolated from five of six nasal swab specimens collected from group II calves. Bovine respiratory syncytial virus was isolated from four of six nasal swab specimens collected from group III calves. No other viruses were isolated from any of the calves.

F. Gross Pathologic Findings

Macroscopic lesions were not observed in group I (Figure 9). In groups II and III, gross lesions were limited to the lungs with no lesions being observed in the upper respiratory tract. The lesions were more pronounced in group II calves (Figure 10) and were characterized by red, consolidated areas most often located in the right and left cranial and right middle lung lobes. Mild to moderate interlobular edema was observed in the caudal lung lobes. An emphysematous bulla was observed in a caudal lung lobe of one calf. Although only 1 lobe was entirely consolidated (middle of calf #12), 7 of 48 lobes of group II calves displayed > 50% consolidation.

Gross lesions in group III calves were minimal and consisted primarily of red to purple, small (0.5 - 5 cm x 0.5 - 4 cm), firm areas (Figure 11). Two calves had no detectable gross lesions.

G. Microscopic Pathologic Findings

Minimal histologic lesions were observed in the respiratory tract of group I calves. Occasional small perivascular aggregates of lymphocytes and macrophages were present in one lung lobe of one calf and a mild focal subacute suppurative pneumonia was found in one lung lobe in each of two calves.

Histologic lesions in all group II calves were characterized by a moderate multifocal interstitial pneumonia, and a moderate multifocal necrotizing or proliferative bronchiolitis. A mild to moderate multifocal bronchopneumonia was observed in 3 of 6 calves. Multinucleate syncytial cell formation was present in bronchiolar and alveolar lumina in consolidated areas in 4 of 6 calves (Figure 12). Mild to moderate hyperplasia of lining epithelial cells were present in multiple bronchioles. Bronchioles within areas of consolidation contained small to moderate quantities of exudate consisting mainly of neutrophils, macrophages, exfoliating and degenerating epithelial cells, and occasional syncytial cells. Areas of interstitial pneumonia usually were adjacent to bronchioles. Interstitial pneumonia was characterized by mild to moderate thickening of alveolar septa due to edema, infiltration of mononuclear inflammatory cells (macrophages, lymphocytes, and plasma cells), and/or swelling of alveolar lining epithelial cells (Figure 13). In a few areas there was mild proliferation of type II pneumocytes. The tracheobronchial lymph nodes demonstrated moderate lymphoid hyperplasia. Examination of sections of nasal turbinates and trachea revealed no lesions.

In group III calves, 3 of 6 calves had no significant histologic changes. Only one calf displayed lesions that could be attributable to BRSV and consisted of minimal multifocal interstitial pneumonia with syncytial cell formation. One calf displayed pooling of neutrophils in the pulmonary vessels in small areas, but the lungs were otherwise normal histologically. Two calves displayed mild multifocal interstitial pneumonia. Mild focal atelectasis was present in occasional lobules in these calves, but since inflammatory changes were lacking, this was most likely due to inadequate formalin perfusion. Two calves were observed to have moderate lymphoid hyperplasia of tracheobronchial lymph nodes. One calf had a moderate diffuse subacute rhinitis.

H. Pneumonic Lung Volumes

A significant difference was observed in pneumonic lung volume among groups ($P < 0.01$) (Figure 14). Using the least significant difference (LSD) test for planned comparisons, group II calves pneumonic volumes were significantly higher at the 99% level compared to groups I and III.

I. Immunoperoxidase Staining

BRSV antigen was detected by avidin-biotin complex immunoperoxidase staining in all group II calves but not detected in group I or group III calves. Bovine respiratory syncytial virus antigen was detected diffusely throughout the lungs of group II calves, however, some calves had only occasional peroxidase-

staining cells while others had numerous stained cells. Bovine respiratory syncytial virus antigen was most frequently detected in the epithelial lining of bronchioles (Figure 15), in the lumens of bronchioles, and in alveolar lumens and surrounding parenchyma. Syncytial cells were present within bronchiolar mucosa (Figure 16) and in alveoli surrounding bronchioles (Figure 17).

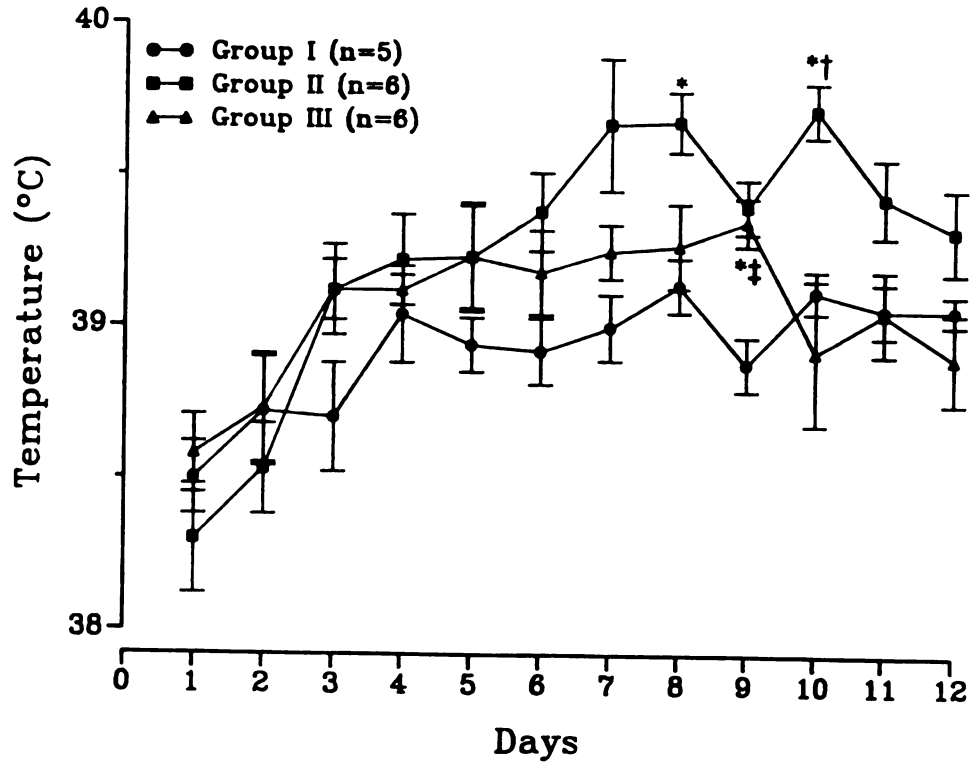
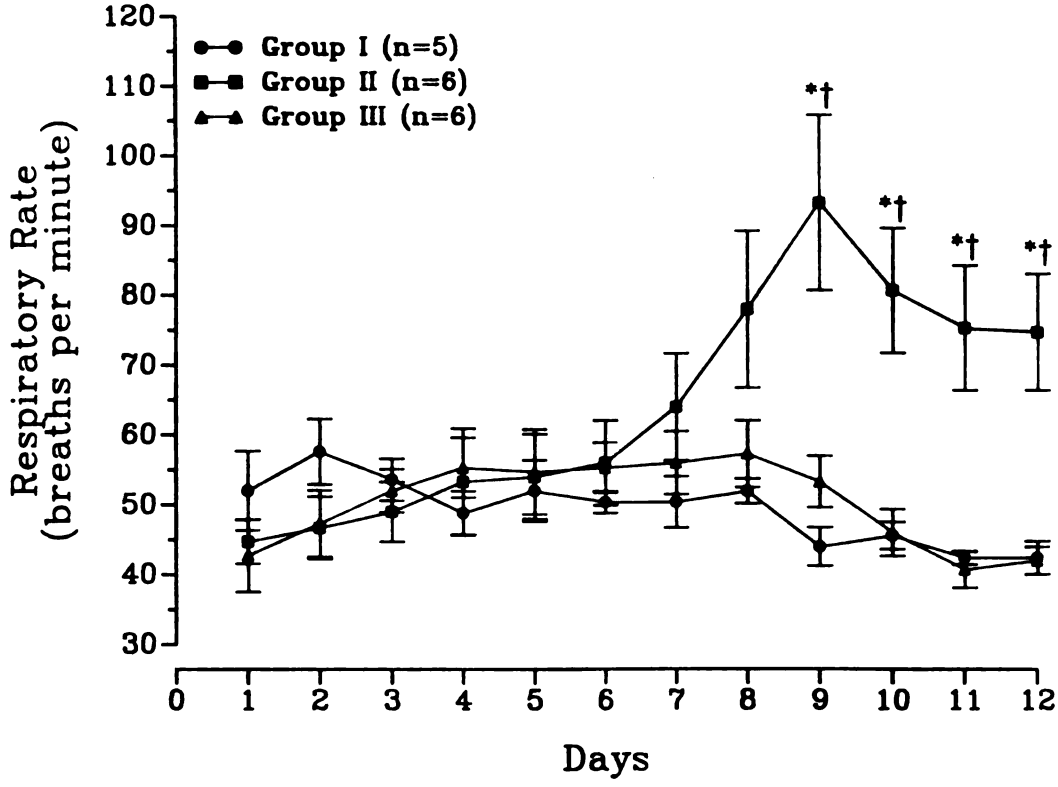


Figure 1. Mean rectal temperature (°C) (± SE) in groups I, II, and III. Group I calves were colostrum deprived and inoculated with tissue culture media. Group II calves were colostrum deprived and inoculated with BRSV. Group III calves were colostrum fed and were inoculated with BRSV. Calves were inoculated on days 3 - 6.



* sig. dif. between Group I and Group II
† sig. dif. between Group II and Group III

Figure 2. Mean respiratory rate (breaths/min) (± SE) of groups I, II, and III. Group I calves were colostrum deprived and inoculated with tissue culture media. Group II calves were colostrum deprived and inoculated with BRSV. Group III calves were colostrum fed and were inoculated with BRSV. Calves were inoculated on days 3 - 6.

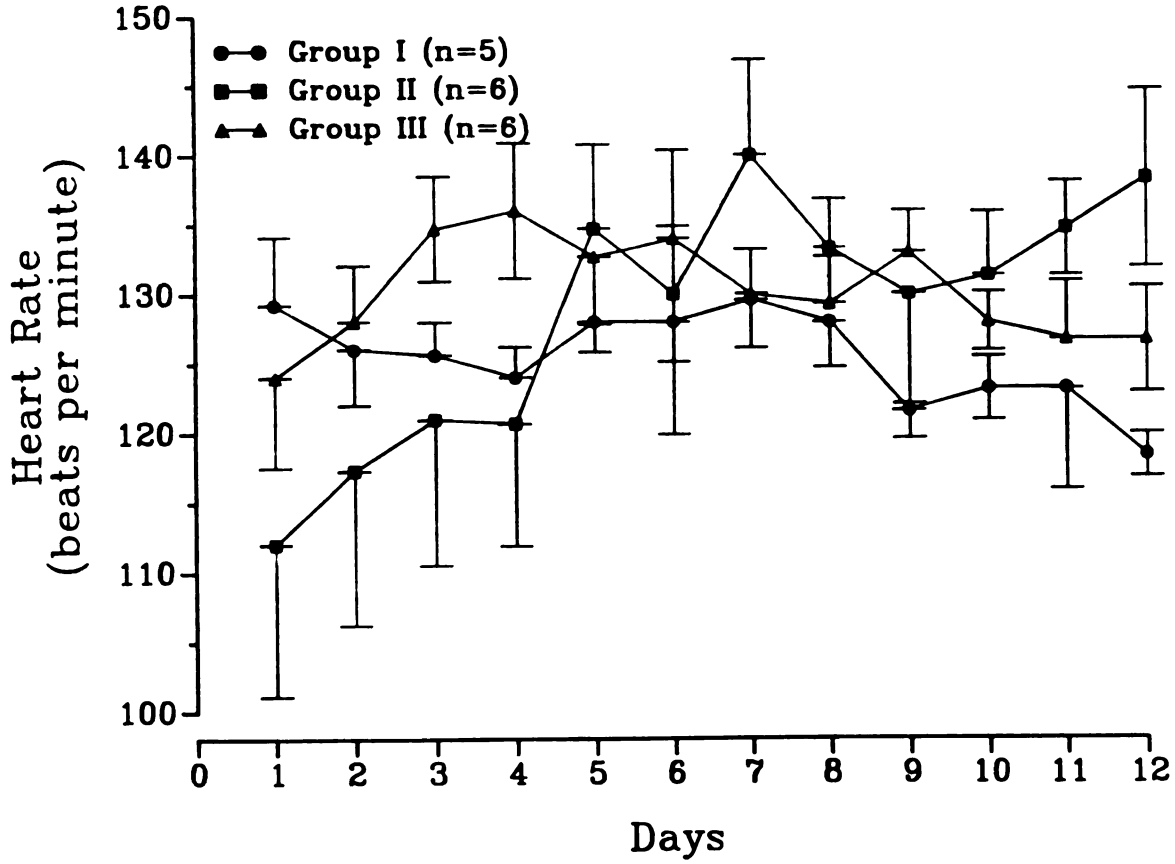
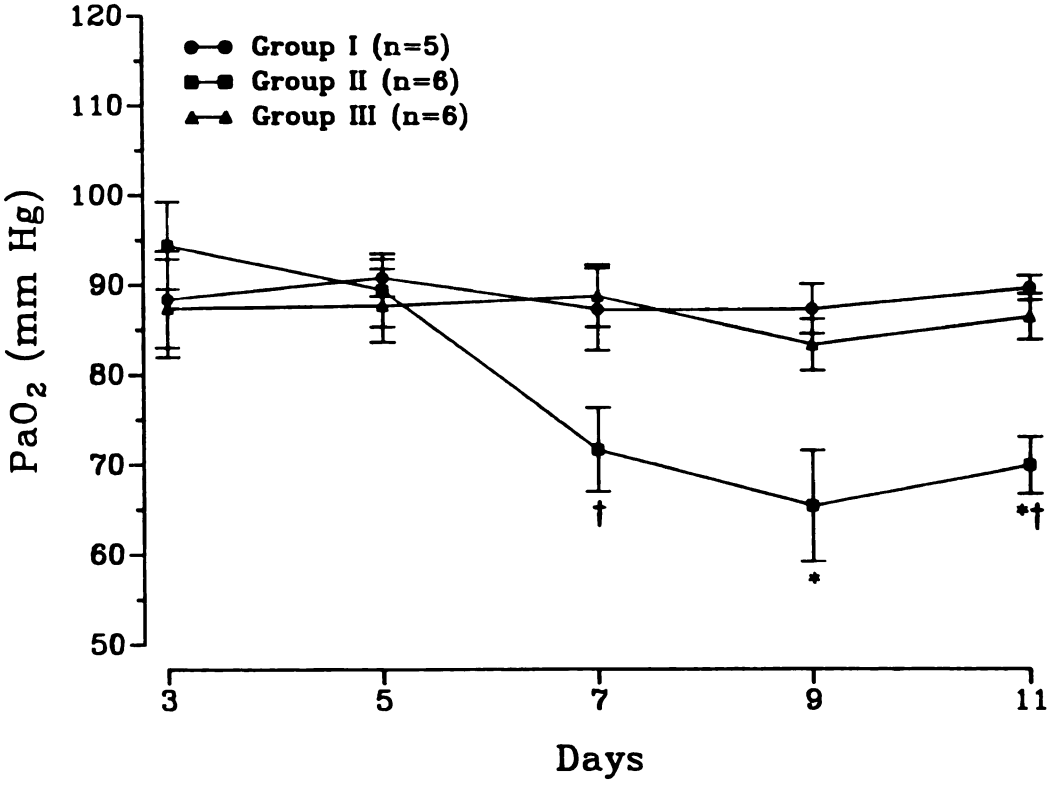


Figure 3. Mean heart rate (beats/min) (± SE) of groups I, II, and III. Group I calves were colostrum deprived and inoculated with tissue culture media. Group II calves were colostrum deprived and inoculated with BRSV. Group III calves were colostrum fed and were inoculated with BRSV. Calves were inoculated on days 3 - 6.



* sig. dif. between Group I and Group II
† sig. dif. between Group II and Group III

Figure 4. Mean arterial oxygen tension (P_aO_2) (± SE) in groups I, II, and III. P_aO_2 was corrected for rectal temperature. Group I calves were colostrum deprived and inoculated with tissue culture media. Group II calves were colostrum deprived and inoculated with BRSV. Group III calves were colostrum fed and were inoculated with BRSV. Calves were inoculated on days 3 - 6.

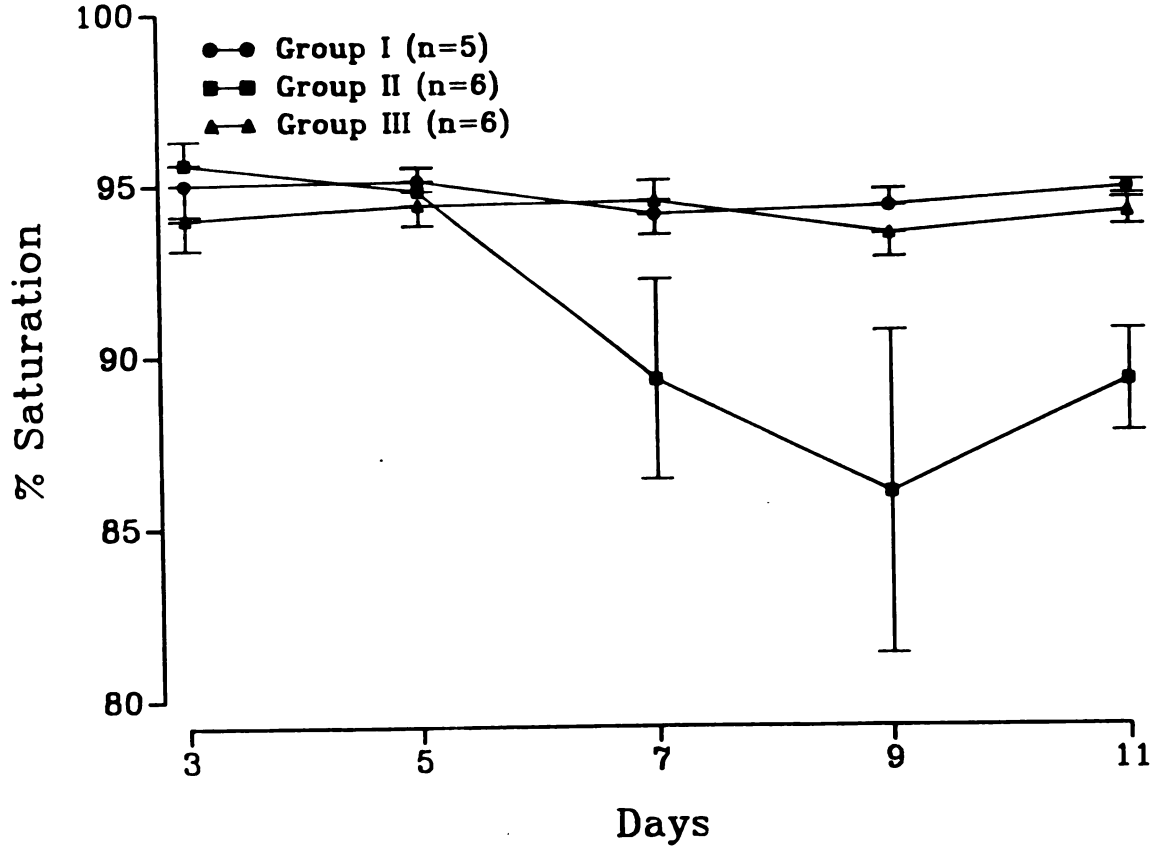


Figure 5. Mean per cent saturation of hemoglobin (± SE) in groups I, II, and III. Group I calves were colostrum deprived and inoculated with tissue culture media. Group II calves were colostrum deprived and inoculated with BRSV. Group III calves were colostrum fed and were inoculated with BRSV. Calves were inoculated on days 3 - 6.

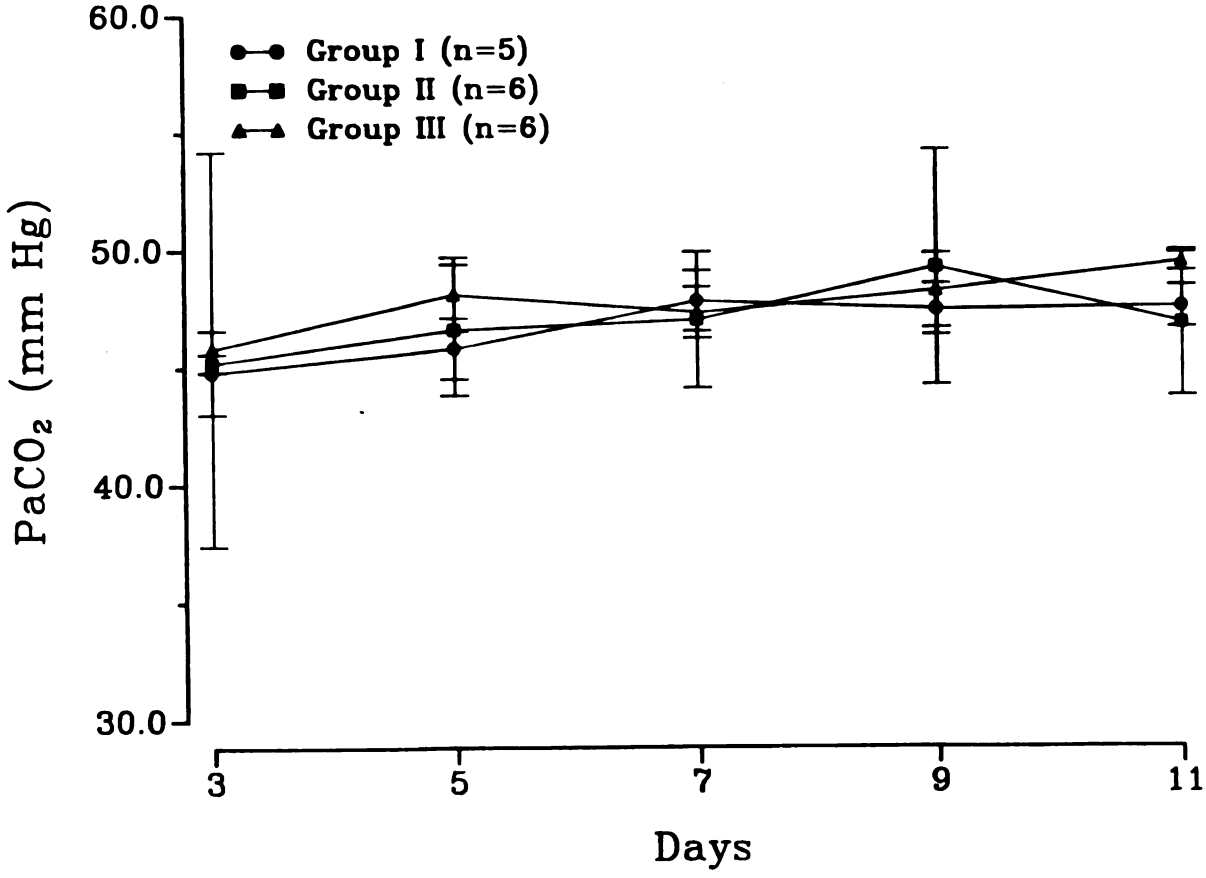
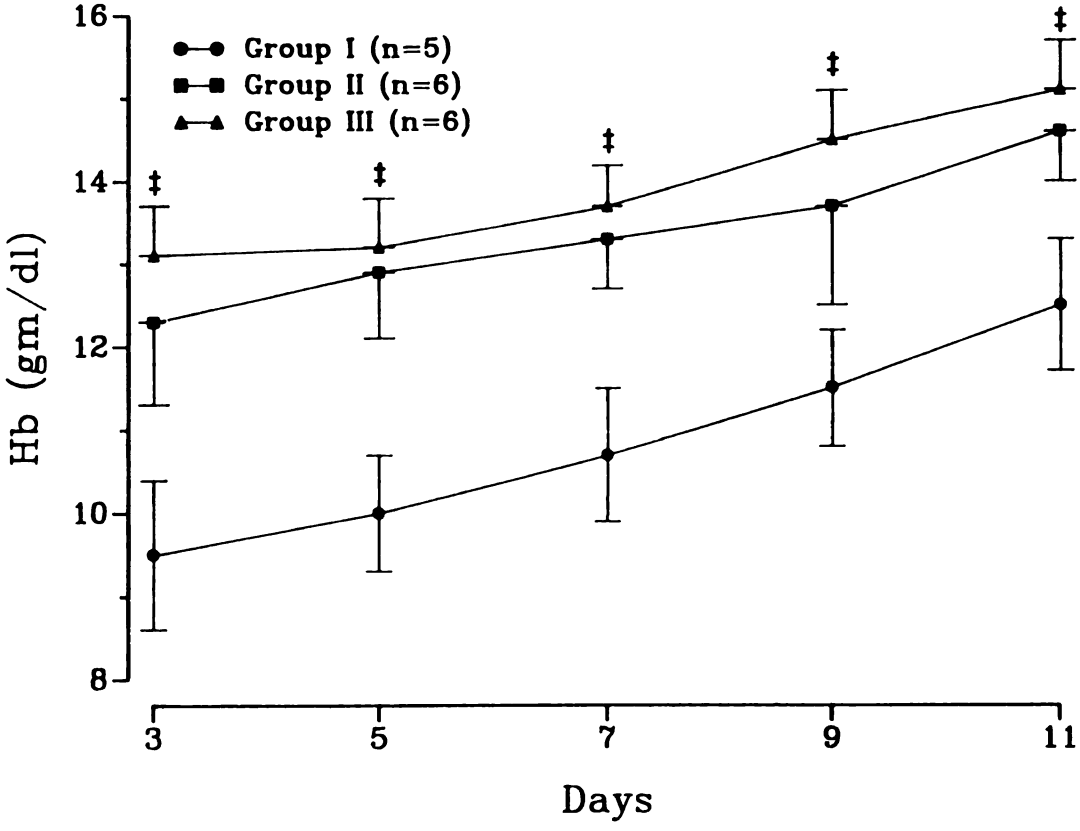


Figure 6. Mean arterial carbon dioxide tension (P_aCO_2) (± SE) in groups I, II, and III corrected for rectal temperature. Group I calves were colostrum deprived and inoculated with tissue culture media. Group II calves were colostrum deprived and inoculated with BRSV. Group III calves were colostrum fed and were inoculated with BRSV. Calves were inoculated on days 3 - 6.



‡ sig. dif. between Group I and Group III

Figure 7. Mean hemoglobin levels (± SE) in groups I, II, and III. Group I calves were colostrum deprived and inoculated with tissue culture media. Group II calves were colostrum deprived and inoculated with BRSV. Group III calves were colostrum fed and were inoculated with BRSV. Calves were inoculated on days 3-6.

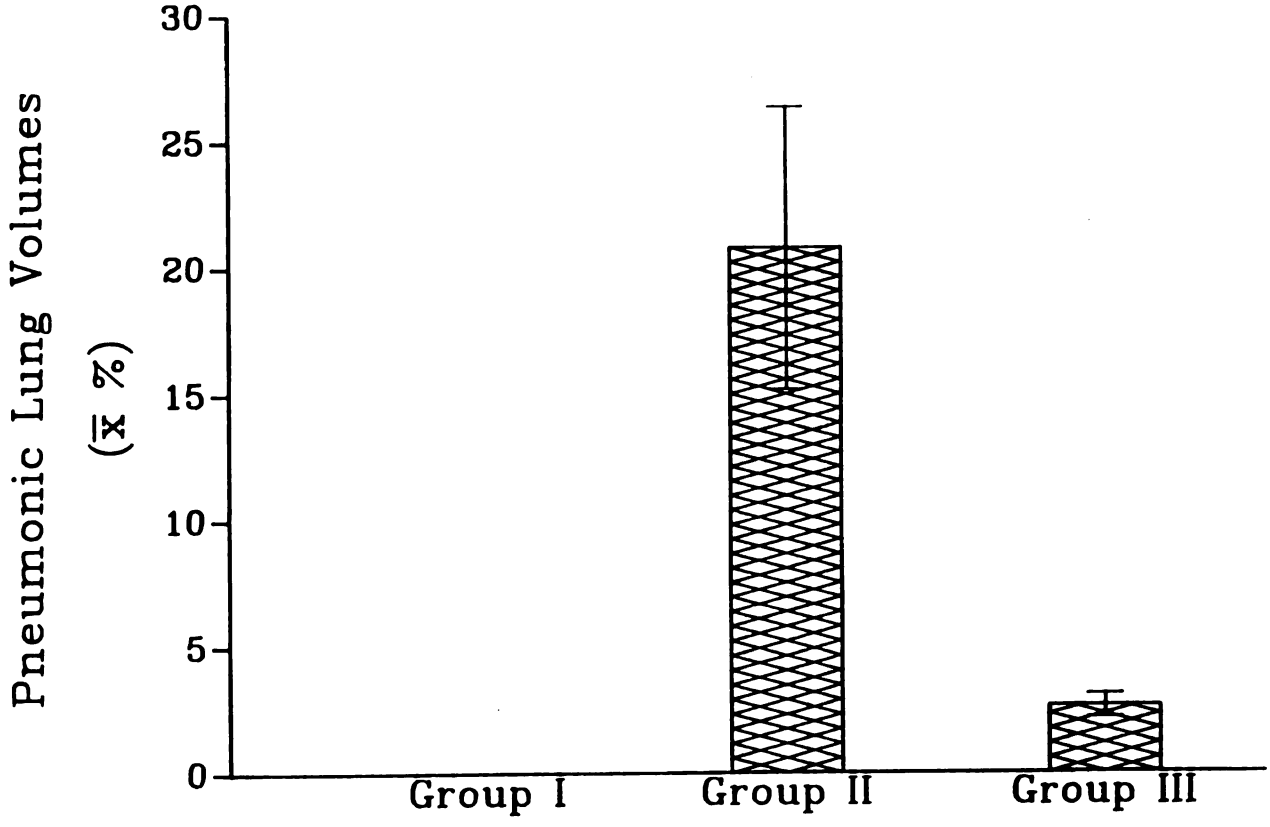


Figure 8. Mean per cent pneumonic lung volume in groups I, II, and III. Group I calves were colostrum deprived and inoculated with tissue culture media. Group II calves were colostrum deprived and inoculated with BRSV. Group III calves were colostrum fed and were inoculated with BRSV. Calves were inoculated on days 3 - 6.

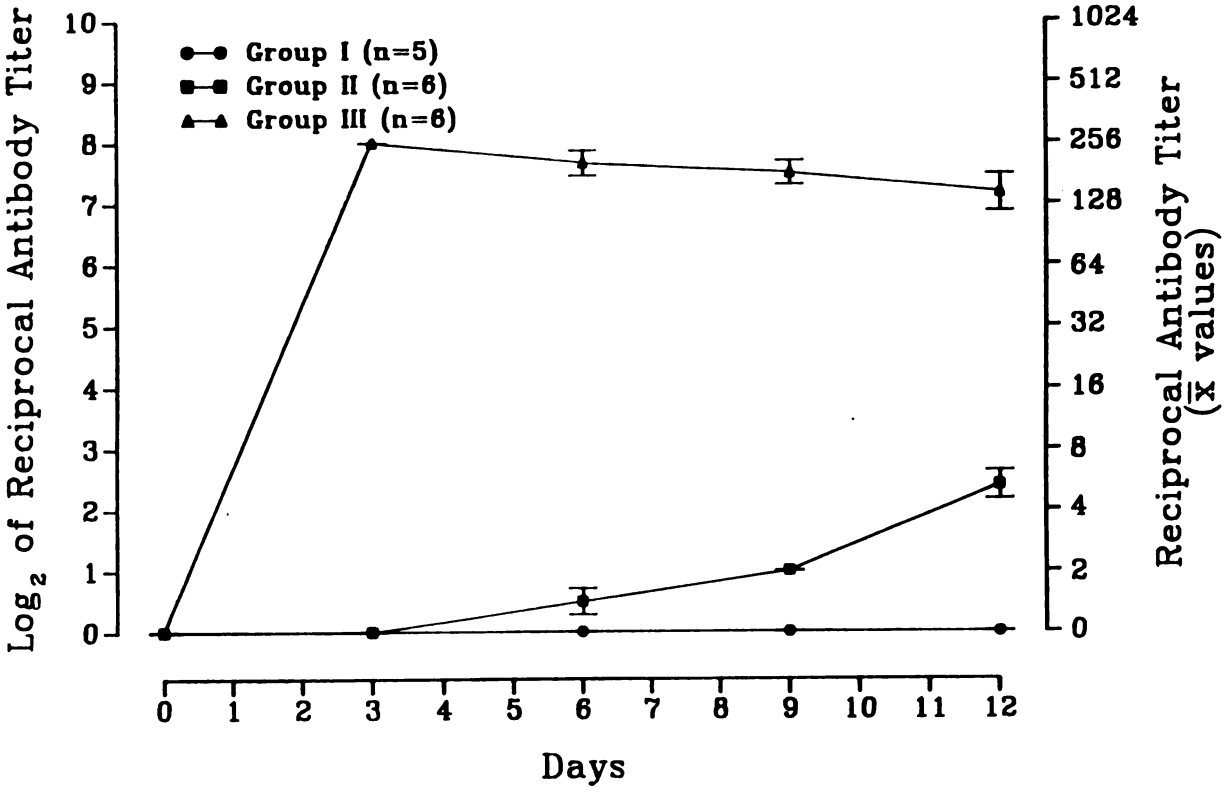


Figure 9. Geometric mean serum antibody titer to BRSV. Group I calves were colostrum deprived and inoculated with tissue culture media. Group II calves were colostrum deprived and inoculated with BRSV. Group III calves were colostrum fed and were inoculated with BRSV. Calves were inoculated on days 3 - 6.

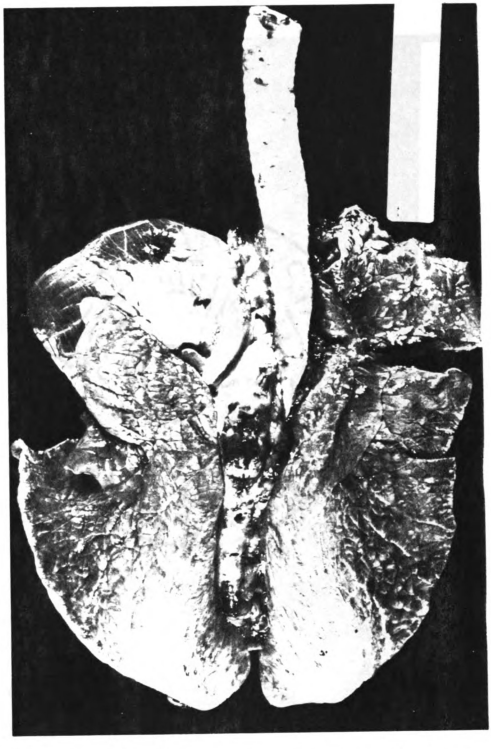


Figure 10A. Dorsal (A) view of lungs from a group I calf. Group I calves were colostrum deprived and inoculated with tissue culture media on days 3 - 6. No gross lesions were present.

Figure 10B. Ventral (B) view of lungs from a group I calf. Group I calves were colostrum deprived and inoculated with tissue culture media on days 3 - 6. No gross lesions were present.

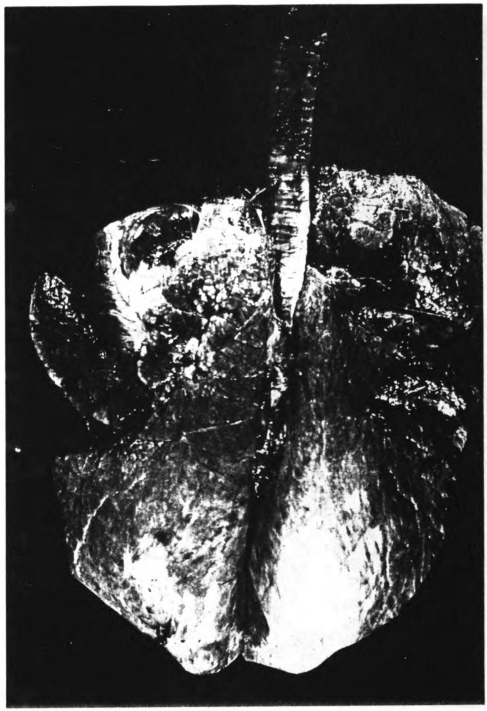


Figure 11A. Dorsal (A) view of lungs from a group II calf. Group II calves were colostrum deprived and inoculated with BRSV on days 3 - 6. Red to red to purple firm areas are noted throughout the lung. Interlobular septal edema as well as an emphysematous bulla (arrow) are present.

Figure 11B. Ventral (B) view of lungs from a group II calf. Group II calves were colostrum deprived and inoculated with BRSV on days 3 - 6. Red to red to purple firm areas are noted throughout the lung. Interlobular septal edema as well as an emphysematous bulla (arrow) are present.

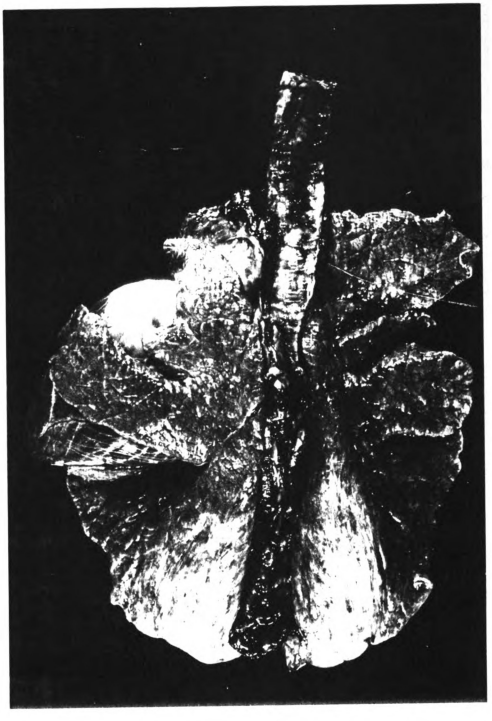


Figure 12A. Dorsal (A) view of lungs from a group III calf. Group III calves were colostrum fed and were inoculated with BRSV on days 3 - 6. Only small, red to purple, firm areas are present (indicated by arrows).

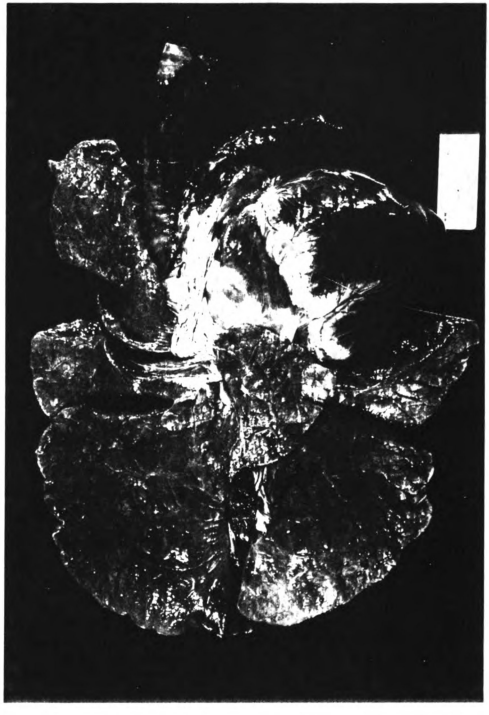


Figure 12B. Ventral (B) view of lungs from a group III calf. Group III calves were colostrum fed and were inoculated with BRSV on days 3 - 6. Only small, red to purple, firm areas are present (indicated by arrows).

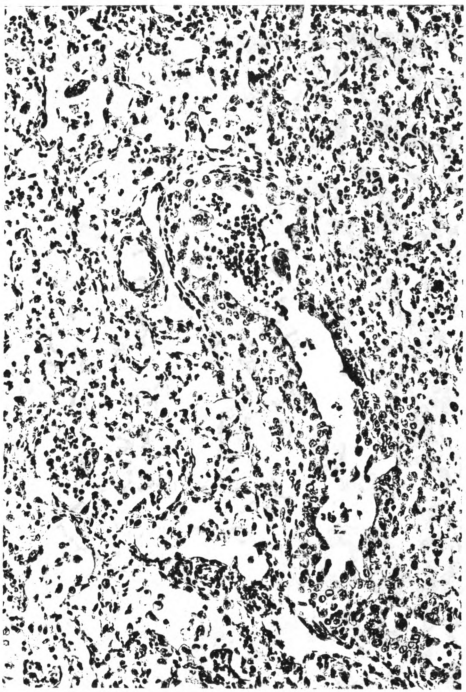


Figure 13. Photomicrograph of syncytial cells arising from mucosal epithelium of a bronchiole in a group II calf. Group II calves were colostrum deprived and inoculated with BRSV on days 3 - 6. A neutrophilic exudate is present in the lumen and a mixed inflammatory infiltrate is present in the surrounding parenchyma, consisting of neutrophils and mononuclear cells. A syncytial cell is also present (arrow). H and E stain. X 330.

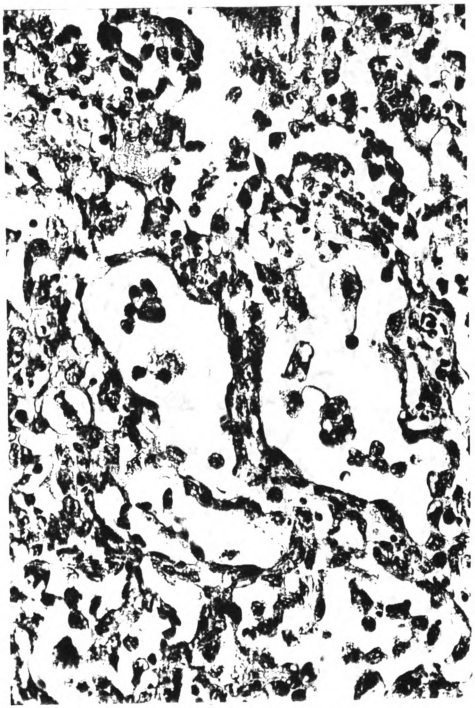


Figure 14. Photomicrograph of alveoli with macrophages and syncytial cells present in the lumen of a group II calf. Group II calves were colostrum deprived and inoculated with BRSV on days 3 - 6. Scattered inflammatory cells are present in surrounding alveoli. H and E stain. X 660.

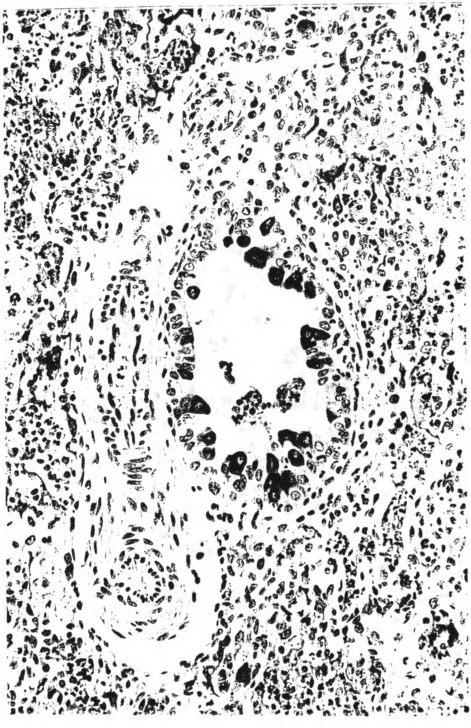


Figure 15. Photomicrograph of a bronchiole and an arteriole in a group II calf. Group II calves were colostrum deprived and inoculated with BRSV on days 3 - 6. BRSV antigen is demonstrated in epithelial cells present in the bronchiole lumen by immunoperoxidase staining. X 270.

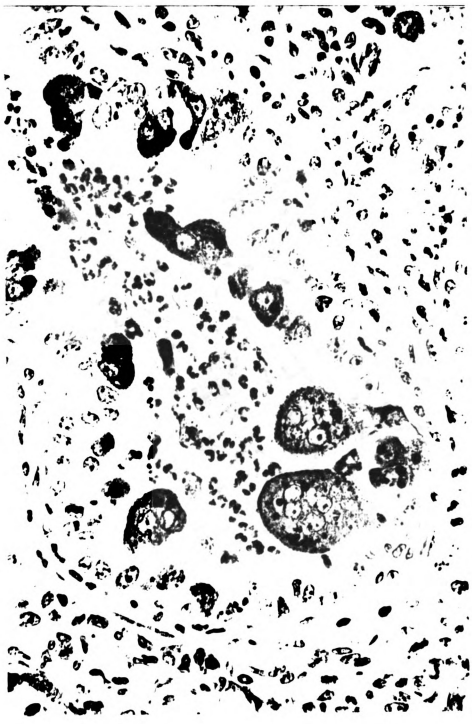


Figure 16. Photomicrograph of bronchiole in a group II calf. Group II calves were colostrum deprived and inoculated with BRSV on days 3 - 6. BRSV antigen is demonstrated in epithelial cells and multiple syncytial cells arising from the mucosal epithelium. Neutrophilic exudate is present in the lumen. Avidin-biotin immunoperoxidase stain. X 660.

Figure 17. Photomicrograph of syncytial cells in center of alveolar interstitium in a group II calf. Group II calves were colostrum deprived and inoculated with BRSV on days 3 - 6. Disruption of alveolar architecture is present as well as necrotic debris throughout. Avidin-biotin immunoperoxidase stain. X 660.

IX. SUMMARY AND CONCLUSIONS

Unlike cotton rats, mice, and ferrets which have also been used to study BRSV and HRSV, calves served as a more appropriate animal model for RSV infections in that they are naturally infected with BRSV. Additionally, calves offer the unique opportunity to study the role of passive immunity in RSV infections in that the presence or absence of passive immunity can be controlled through the feeding of colostrum. Not only are calves naturally infected with RSV, but BRSV infections share many similarities with HRSV infections which include epidemiologic, clinical, pathologic, and possibly pathogenetic mechanisms.

Bovine respiratory syncytial virus infection of group II calves resulted in the production of clinical signs and respiratory tract lesions similar in severity to those described from naturally occurring infections.[7,8,41] The majority of previous attempts to experimentally reproduce respiratory disease with BRSV have resulted in the production of only mild disease.[32,87,88] Two previous studies of experimental BRSV infection which utilized low passage level virus and a combined respiratory tract route of infection (intranasal and intratracheal) were successful in producing severe respiratory disease and respiratory tract lesions.[77,78] Our infection protocol was adapted from these two studies and our results confirm their previous findings. The production of severe disease appears to be related to using a combined respiratory route of inoculation over a four day period in conjunction with a low passage BRSV.

Signs of respiratory tract disease following BRSV infection were most severe in group II calves as demonstrated by the higher rectal temperatures, heart rates, and respiratory rates in comparison to groups I and II (Figures 1-3). These clinical signs corresponded with those produced in naturally occurring BRSV infections.

Arterial blood gas tensions displayed significant differences between group II calves as compared to groups I and III, with P_aO_2 values being significantly lower in group II calves and indicative of hypoxemia (Figure 4). Similar findings have been reported from other studies of natural BRSV infection in calves.[89-92] Hypoxemia has also been reported in human infants with HRSV.[93] In the present study, the most severe clinical signs and the most striking respiratory rate changes were observed in group II calves, the group which had significantly lower P_aO_2 values than groups I and III (Figure 4). Arterial oxygen tension values in group III calves closely resembled those of group I (control group) (Figure 4), suggesting less severe lung involvement and, thus, a protective effect of maternally-derived antibodies on BRSV infection.

Hypercapnea has been reported, but has not been a consistent finding in infants and calves with RSV infections.[91,93] Respiratory syncytial virus-induced hypercapnea was not observed in our experiment (Figure 6) as had been observed in other studies.[92,93] The decrease in P_aO_2 in the presence of a constant P_aCO_2 was probably the result of a mismatching of ventilation and perfusion. The difference in slope of the oxyhemoglobin and carbon dioxide diffusion curves and the greater solubility of CO_2 account for the impaired O_2 exchange and normal CO_2 exchange

in the lung. An increase in ventilation (only respiratory rate was measured and observed to increase in group II calves) allows removal of CO_2 so that P_aCO_2 values remain normal, while P_aO_2 values remain relatively unchanged. The O_2 saturation levels measured in calves were significantly lower in group II calves over time and they plotted similarly to P_aO_2 values within the same group (Figure 5). This result can be explained by the fact that O_2 saturation levels are dependent on P_aO_2 when P_aCO_2 is constant.[94] Hemoglobin levels increased significantly over time in all groups, but were significantly different only between groups I and III. Reasons for this result are uncertain, although it is noted that there was a significant difference between groups I and III present on day 1 prior to inoculations with BRSV. A possible explanation for the increase in hemoglobin over time may be that all calves were anemic at birth and resolved this problem over the next eleven days, accounting for an increase in hemoglobin levels. We offer no explanation for the differences between groups in hemoglobin levels.

All calves in groups I and II were considered to have failure of passive transfer of immunuglobins (IgG levels < 500 mg/dl),[95] which was expected as neither group was fed colostrum. Adequate passive transfer of immunoglobins is thought to be indicated by IgG levels greater than 1200 mg/dl.[95] Adequate passive transfer of immunoglobins was observed in four group III calves, while the other two calves in group III were found to have partial passive transfer of immunoglobins (IgG levels between 500 - 1200 mg/dl).

All calves had undetectable levels of serum antibodies to BRSV on day 1 (Table 2), which was expected as calves had not received colostrum prior to sample collection. Antibodies to BRSV remained undetectable in group I, while group II calves seroconverted to BRSV following infection and group III calves showed the highest level of BRSV specific antibody following the ingestion of colostrum. The above findings were expected in that group I was inoculated with tissue culture media, group II was infected in the absence of BRSV-specific antibody, and group III was fed colostrum which accounted for their high levels of BRSV specific antibody. Passive immunity in group III calves suppressed an active immune response as observed by the lack of increase in BRSV specific antibody levels following BRSV inoculation as has been observed in previous studies.[19,22] In group III, BRSV antibody levels remained stable or declined over the study period after colostrum consumption (Table 2), which would be expected if all antibodies were of maternal origin prior to inoculation with BRSV.

Neither isolation of, nor seroconversion to IBRV, BVDV, or PI_3 virus was observed in any of the groups, although antibody titers to these viruses rose in group III calves following the ingestion of colostrum and subsequent absorption of specific antibodies to these viruses. Virus isolation was attempted on day 8 and BRSV was isolated in 5 of 6 group II calves, 4 of 6 group III calves, and no group I calves. These results were expected as group I calves were not inoculated with BRSV and group II and III calves were inoculated with BRSV. It does not appear that colostrum feeding had an effect on BRSV isolation in group III calves.

Gross lesions in the respiratory tract were not observed in group I calves (Figure 10). Group III calves had gross lesions (Figure 11) in the respiratory tract suggestive of BRSV infection, but lesions were less extensive than those observed in group II calves (Figure 12). Lesions identified in group II calves were similar to those previously described in BRSV experimental studies,[77,78] and in other naturally occurring BRSV infections.[2,6,7,8,9,96] These lesions included consolidation of lobules, interstitial and interlobular edema, and were similar to those reported from fatal HRSV infections in infants.[10,11] Significant differences in per cent pneumonic lung volume ($P < 0.01$) were seen among groups (Figure 8) with only minimal lesions occurring in group III (colostrum-fed) calves and no lesions in group I (control) calves. While calves of group II had pneumonic lung volumes of greater than 20%, group III calves had less than 5% pneumonic lung volume. Lesions were observed in all lung lobes, although a cranioventral distribution predominated. A previous study of experimental BRSV infections in calves inoculated by a combined respiratory tract route identified areas of pneumonia in cranial, middle, accessory, and cranial portions of caudal lung lobes, while a similar experimental study was only able to reproduce consolidation in cranial and middle lobes.[77,78] In contrast to our study, the latter study and others were only able to produce emphysema and edema in the caudal lung lobes.[45,78,97]

Microscopic lesions in group II calves resembled lesions reported previously in both experimental and naturally occurring BRSV infections.[6,8,78] Lesions in group II calves were characterized by a multifocal interstitial pneumonia with moderate

bronchiolitis. Syncytial cell formation was common in bronchioles of group II calves, but uncommon in group III with only one calf having syncytial cells present. Other calves in group I and most group III calves had no lesions attributable to BRSV, again suggesting that maternally-derived antibodies specific to BRSV may be responsible for the protection displayed by group III calves.

Formalin-fixation of the lungs by perfusion at uniform pressure through the pulmonary artery and trachea was required to obtain accurate volumetric measurement of pneumonic lung and total lung volumes. As these procedures had to be accomplished immediately after euthanasia with an intact pleura, collection of lung tissue samples for bacterial culture and virus isolation was not done. Volumetric determination of pneumonic lesions via serial slicing and computer data digitalization allowed for a quantitative assessment of lesions and comparison of mean pneumonic lung volume among groups. Although a previous study of *Haemophilus somnus* pneumonia in calves used a similar procedure, no other experimental infection studies with BRSV have used this method of quantitating lung lesions.[98] While lungs were perfused only through the vasculature after flushing with saline in the latter study, we found it more effective to exclude the saline flush and perfuse immediately with formalin simultaneously through the trachea and vasculature. This modification allowed for more uniform perfusion of the lungs.

Immunoperoxidase staining, using antibodies to HRSV which cross-react with BRSV, was used for detection of the BRSV antigen in lung tissue.[99] Bovine

respiratory syncytial virus antigen was detected only in group II calves. As with pneumonic lesions, BRSV antigen was distributed throughout the lung but was particularly concentrated in the cranial lung lobes. While certain calves had only scattered positive staining cells, others had numerous positive staining cells. Other natural infections have reported that BRSV antigen was found only in cranioventral and not caudodorsal portions of the lungs.[45,97] In these latter studies, emphysema and alveolar edema were the main lesions noted in the caudodorsal region of the lungs, with bronchitis and bronchiolitis also being observed in the cranioventral portion of the lungs. Reasons for finding virus-associated lesions only in the cranioventral portion of the lungs included a possible endobronchial dissemination of virus and a possible gravitational role or aerosol transmission pattern for this process, while suggesting a different pathogenetic mechanism and possibly even an immune-mediated mechanism for the lesions in the caudodorsal lung lobes.[45] In the current experimental study, the finding of BRSV antigen by immunoperoxidase staining throughout the lungs of group II calves as well as accompanying lesions of bronchiolitis, bronchitis, interstitial and interlobular edema tend to support an airway dissemination of the virus.

Although BRSV antigen was not detected in group III calves by immunoperoxidase staining, the lungs of one calf did contain syncytial cells histologically, suggesting that virus was present in the lower respiratory tract. There was no correlation in group III calves among % pneumonic lung volume, IgG levels, and isolation of virus. The lack of BRSV staining cells by immunoperoxidase in

group III calves indicated that maternally-derived antibodies may have been involved in neutralizing the virus, preventing viral adsorption or limiting viral replication and spread, thus allowing for protection in the lower respiratory tract and modifying the severity of disease. However, it does appear that infection was established in some of the group III calves as seen by the presence of syncytial cells in one calf and the isolation of virus from four calves. It should be noted, however, that a severe challenge with BRSV was utilized over a four day period and colostrum still afforded protection. BRSV antigen was demonstrated in histologic sections of the lungs of all group II calves by avidin-biotin immunoperoxidase staining, however. While virus was isolated from most group III (colostrum-fed) calves, no antigen was detected by immunoperoxidase staining of lung tissue, indicating that colostrum-derived passive immunity offered some protection against BRSV infection of the lower respiratory tract. Conversely, calves in group II which were classified as having failure of passive transfer (IgG levels < 500 mg/dl) had detectable BRSV antigen in lung tissue by immunoperoxidase staining.

Previous studies have utilized subjective scoring methods for quantitative and qualitative measurements of pneumonia. Other studies have utilized blood gas tensions to assess the severity of disease, but have not correlated the findings with postmortem examination of lungs.[89,90,91] Our study utilized quantitative clinical parameters and blood gas tensions to assess severity of disease and then used a more precise method to quantitate pneumonic lesions. These data in conjunction with immunoperoxidase staining for BRSV antigen and virus isolation results,

enabled us to accurately compare treatment groups and to thoroughly define BRSV-induced disease. It should be noted that the calves used in the current study were all less than two weeks of age when the study was completed, and passive immunity to BRSV may not modify the disease severity to the same extent in older colostrum fed calves.

Strict isolation procedures were followed in this study, as approximately one third of animals with viral pneumonia are secondarily infected with organisms from normal upper respiratory tract flora.[100] Calves in this study were not gnotobiotic and likely the upper respiratory tract was colonized by bacterial pathogens involved in bovine respiratory disease. It appears that maintenance of calves on an antibiotic successfully prevented secondary bacterial infection of lungs following BRSV inoculation based on the lack of bacteria observed on microscopic examination of the lungs. Immunoglobins are a part of the lungs secretory defense, and with two-thirds of the calves being colostrum deprived this component of defense was lacking.[94] Maximum depression of the lungs bactericidal capabilities is seen seven days after viral infection and is thought to be due to the destruction of the mucociliary clearance system, an increase in alveolar exudate providing a media for bacterial growth, and a decrease in the phagocytic capacity of alveolar macrophages.[100,101] Our calves were necropsied nine days after the first BRSV inoculation and six days after the last inoculation, allowing adequate time for a secondary bacterial pneumonia to occur. Since this study was concerned only with effects of virus infection, control for secondary bacterial pneumonia was necessary.

For the above reasons, an antibiotic (ceftiofur sodium) which has activity against the most common bovine respiratory tract bacterial pathogens was given to the calves systemically.

Passive immunity may not prevent infection by BRSV, as 4 of 6 calves in group III had BRSV recovered by virus isolation. However, passive immunity may modify the severity and/or the extent of the infection and thus the resultant disease as evident by results of P_aO_2, O_2 saturation, respiratory rate, and temperature in group III calves as compared to group II calves. Data from group III calves more closely resembled that of group I (control) calves, indicating that passively derived immunity suppressed the severity of BRSV-induced disease.

We are assuming that the maternal antibodies to BRSV were responsible for the protection offered by the colostrum, however the possibility can not be excluded that there may have been another factor or factors in colostrum responsible for its protective effect. Possibilities for these factors could include transfer of cell-mediated immunity specific to BRSV.[54,55]

The role of passive immunity in HRSV and BRSV infections has been previously studied. In HRSV infections, maternal antibody function is controversial as some studies report that high levels of maternally-derived antibodies offer protection early in life while others report no protection offered by maternal antibodies.[17,21,37,57] In all but one study, maternally-derived antibodies specific for BRSV have been reported to have no protective effect against BRSV-induced disease.[19,22,23,71,72,78] The only study to demonstrate a protective role for maternally-

derived antibody was a recent epidemiologic field study of natural BRSV infections found that when calves younger than three months of age became infected with BRSV, the frequency and severity of the disease was inversely related to the level of specific IgG in their acute phase sera.[23] In this epidemiologic study BRSV-specific serum IgG titers in the calves were assumed to be maternally derived. Passive immunity derived from colostrum has been demonstrated to be protective for other calfhood diseases including rotavirus, coronavirus, and coliform diarrhea. Passive immunity has demonstrated a decrease in mortality for respiratory and enteric diseases and a decrease in morbidity for enteric diseases alone.[102] In the present study, we found that passive immunity to BRSV modified the severity of disease following experimental BRSV challenge.

From the current study it can be concluded that colostrum, as a source of passive immunity, is protective in BRSV infections in calves less than two weeks of age. Further studies are indicated to define cell-mediated protection afforded by BRSV-specific lymphocytes in colostrum which are passively transferred to the neonate. Further studies would also include evaluation of serum samples of colostrum-fed calves for responses to BRSV-specific glycoproteins. Based on our results, possible recommendations for prevention of BRSV infection in calves would include vaccinating dry cows for BRSV and feeding colostrum high in specific immunoglobin levels to BRSV to the calf immediately after birth. This study clearly demonstrates that experimental inoculation of colostrum-deprived calves produced severe clinical BRSV disease, that colostrum was the sole source of BRSV-specific

antibodies, and that these preexisting antibody levels may be responsible for modifying the severity of BRSV disease in calves. This refutes a previous hypothesis of calves and infants which stated that maternal antibodies exacerbate the severity of RSV infections, and supports the one study that found maternal antibodies to offer some protection against BRSV infections.

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