EXPERIMENTAL REPRODUCTION OF RESPIRATORY TRACT DISEASE WITH BOVINE RESPIRATORY SYNCYTIAL VIRUS

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ABSTRACT

EXPERIMENTAL REPRODUCTION OF RESPIRATORY TRACT DISEASE
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A controlled study was undertaken to experimentally reproduce respiratory tract disease with bovine respiratory syncytial virus (BRSV) in one-month-old, colostrum-fed calves. The hypothesized role of persistent infections and viral hypersensitivity reactions in the pathogenesis of BRSV pneumonia was also investigated.

Four groups were inoculated. Single exposure to BRSV was compared with BRSV rechallenge. Response was evaluated by daily monitoring of body temperature, respiratory and heart rate, arterial blood gas tensions, hematocrit, total protein, fibrinogen, and leukocyte count. All calves were necropsied and pulmonary surface lesions were quantitated.

Viral pneumonia was successfully reproduced in each principal group. Disease was not apparent in controls. Significant differences in body temperature, heart rate, respiratory rate, arterial oxygen tension, and pneumonic surface area were observed between control and infected calves. BRSV reinoculation had minimal effect on disease progression. Experimental results did not support persistent infection or viral hypersensitivity as mechanisms of BRSV pathogenesis.

It gives me great pleasure to dedicate this work
to Mother, Father and Shelley.

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INTRODUCTION

Respiratory syncytial virus (RSV) has gained acceptance as an important respiratory tract pathogen in the human and bovine species. Despite successful isolation of this virus in goats (Smith et al., 1979) and sheep (Lea Master et al., 1983), respiratory tract disease due to RSV remains poorly defined in these species. Respiratory syncytial virus was initially isolated in 1956 during an outbreak of upper respiratory disease in a chimpanzee colony and was originally termed the "Chimpanzee Coryza Agent" (Morris et al., 1956). The following year, an identical agent was recovered from infants with lower respiratory illness and the virus was subsequently renamed respiratory syncytial virus in recognition of its characteristic cytopathic effect in tissue culture (Chanock et al., 1957). Respiratory syncytial virus infection in cattle was suggested by the discovery of an inhibiting agent in bovine sera which diminished syncytia formation in cell cultures inoculated with human respiratory syncytial virus (HRSV) (Taylor-Robinson and Doggett, 1963). This inhibitory substance was later shown to be specific antibody (Doggett et al., 1968). Bovine respiratory syncytial virus (BRSV) was initially isolated from cattle in Switzerland during an outbreak of respiratory disease in 1967 (Paccaud and Jacquier, 1970).

Bovine respiratory syncytial virus is classified in the Pneumovirus genus of the Paramyxoviridae family. It is a medium-sized (120-155 nm) (Cutlip and Lehmkuhl, 1979; Al-Darraji et al., 1982b; Trigo et al., 1984), pleomorphic, enveloped virus that contains a single strand of

linear ribonucleic acid. Unlike other viruses in the paramyxovirus family, BRSV lacks neuraminidase and is non-hemagglutinating. Virus infectivity is destroyed by ether, chloroform, trypsin and sodium deoxycholate. The virus is rapidly inactivated at pH3, but relatively stable above pH4. Although viral infectivity is labile at 56°C and 37°C, the virus remains stable at -70°C for several months (Inaba et al., 1973).

Bovine respiratory syncytial virus infection has been reported to produce respiratory tract disease in susceptible cattle populations including nursing and weanling beef calves (Bohlender et al., 1982; Baker et al., 1986b), feedlot steers (Smith et al., 1975; Bohlender et al., 1982; Baker et al., 1986b), dairy calves and heifers (Inaba et al., 1972; Harrison and Pursell, 1985; Baker et al., 1986a), and lactating dairy cows (Pacaud and Jacquier, 1970; Harrison and Pursell, 1985). Clinical signs accompanying an outbreak are variable and may include pyrexia (39.4°C/103°F-42.2°C/108°F), depression, conjunctivitis, lacrimation, nasal discharge, cough, anorexia, salivation, hyperpnea, dyspnea and subcutaneous emphysema and edema. Several investigators have reported a biphasic progression of respiratory signs in association with some BRSV outbreaks involving feedlot cattle (Wellemans et al., 1978; Bohlender et al., 1982; Frey, 1982; Baker et al., 1986b). Following the introduction of the virus into a susceptible herd, morbidity is generally high and some deaths may occur. Pulmonary edema, subpleural and interstitial emphysema with bullae formation, and consolidating pneumonia are characteristic gross findings evident at necropsy. Histopathologically, classic lesions include interstitial pneumonia with necrotizing bronchiolitis and alveolitis, alveolar and bronchiolar epithelial hyperplasia,

multinucleated epithelial syncytia, and alveolar edema and hyaline membrane formation. Regions of acute suppurative bronchopneumonia may be observed if secondary bacterial invasion has occurred.

The pathogenesis of respiratory tract disease due to RSV infection is poorly understood. Numerous hypothetical mechanisms, however, have been advanced. A purely mechanical explanation relates to the virus's tropism for luminal epithelial cells of the peripheral airways. Viral infection and replication stimulates epithelial necrosis, mucosal sloughing, inflammatory infiltrates and edema. Pulmonary defense mechanisms become compromised and secondary bacterial or viral invasion ensues. Bovine respiratory syncytial virus and Pasteurella haemolytica have been shown to possess synergistic pathogenic effects under experimental conditions in lambs (Al-Darraji et al., 1982a,b,c; Trigo et al., 1984b). Similar studies have not been conducted in calves.

Several immunopathologic mechanisms have also been advocated in an attempt to explain RSV pathogenesis (McIntosh and Fishaut, 1980). The pneumonia caused by BRSV in cattle has been attributed to a Type I hypersensitivity reaction in some cases (Bohlender et al., 1982; Frey, 1982). As mentioned previously, some BRSV epizootics display two recognizable clinical stages of disease. The initial stage is generally characterized by mild clinical signs. A secondary phase follows apparent improvement or recovery and features severe respiratory distress and death loss may occur. The initial infection has been proposed to serve as a sensitizing infection with subsequent exposure inducing a hypersensitivity reaction. This biphase pattern of respiratory tract disease has only been described in feedlot calves.

RSV's influence on irritant airway receptors, IgE antibody production (Welliver et al., 1980; Welliver et al., 1981), neutrophil kinetics (Faden et al., 1983), alveolar macrophage function and interferon production may also be involved in disease pathogenesis. These and several additional mechanisms have been reviewed (Frey, 1983; Stott and Taylor, 1985). At this time, all suggested mechanisms remain speculative. In reality, the pathogenesis of RSV infection is probably the result of simultaneous contributions from many of the different proposed mechanisms.

LITERATURE REVIEW - EXPERIMENTAL INFECTIONS

In addition to its importance in veterinary medicine, RSV displays considerable pathogenic influence on the human species. Human respiratory syncytial virus is currently considered to be the major cause of bronchiolitis, pneumonia and fatal respiratory tract disease in infants and young children throughout the world (Chanock et al., 1962; Chanock et al., 1982). As a result, numerous experimental infectivity trials have been conducted with RSV in an attempt to identify a relevant animal model for study of this important human and bovine disease. An appropriate reproducible model would facilitate understanding of disease pathogenesis. Safety and efficacy of experimental vaccines could also be evaluated with an effective model.

Numerous animal species have been experimentally infected with RSV including cattle, sheep, Cebus monkeys (Belshe et al., 1977; Richardson et al., 1978a), chimpanzees, (Belshe et al., 1977, Richardson et al., 1978b), squirrel monkeys, Rhesus monkeys (Belshe et al, 1977), owl monkeys (Richardson et al., 1978B), white-lipped marmosets, mink, chinchilla, African rats, guinea pigs, mice (Coates and Chanock, 1962), ferrets (Coates and Chanock, 1962; Prince and Porter, 1976), hamsters (Collier and Clyde, 1977), and cotton rats (Dreizin et al., 1971). Experimental inoculation has only produced clinical disease in cattle, sheep, chimpanzees, Cebus monkeys and owl monkeys. Pathological lesions have been observed in experimentally infected cattle, sheep, Cebus monkeys, ferrets, hamsters, cotton rats and mice.

Discussion in the following review is devoted to experimental BRSV infections in ruminants. Experimental infections have been divided into the following four categories:

1. Experimental BRSV infections in calves.
2. Experimental BRSV infections in gnotobiotic calves.
3. Experimental BRSV infections in sheep.
4. BRSV and Pasteurella haemolytica synergism in sheep.

Within a category, experimental studies are reviewed in chronological order.

I. Experimental BRSV Infections in Calves.

A large epizootic of acute respiratory disease occurred in Japanese cattle during the months from October 1968 to May 1969. Four isolates of BRSV were recovered and the NMK7 isolate of the virus (eleventh passage) was subsequently used to inoculate a healthy, 6-month-old Japanese Black calf by the intranasal (20 ml of infected culture fluid, $10^{3.8}$ median tissue culture infective doses ($TCID_{50}$)/0.1 ml) and intratracheal (10 ml of infected culture fluid, $10^{3.8}$ $TCID_{50}$/0.1 ml) routes. This inoculation represented the first attempt to reproduce respiratory tract disease in calves with BRSV (Inaba et al., 1972).

Following inoculation, the calf developed an acute respiratory illness resembling the natural disease. Pyrexia (39.8°C/103.6°F-41.3°C/106.3°F), anorexia, depression, lacrimation and serous nasal discharge were reported. Leukopenia was observed on the second day following inoculation. All clinical signs completely resolved by nine days post-infection. Virus was recovered from nasal secretions on the fifth, sixth, seventh, ninth and tenth days post-inoculation. The calf developed neutralizing antibody against the virus by two weeks post inoculation.

Early in 1971, BRSV was isolated from a herd of Iowa feedlot cattle experiencing acute respiratory disease (Smith et al., 1975). This isolate, identified as FS1-1, was later utilized in an experimental infectivity study involving 5 calves. The virus had been passaged 18 times in cell culture prior to inoculation. The inoculum was aerosolized intranasally to each animal. Each calf received 4 ml of inoculum containing approximately 4000 PFU (plaque-forming units) of virus.

Clinical signs of illness became apparent in some calves on the fourth day post-inoculation. Three calves displayed anorexia, depression, serous nasal discharge and polypnea. Two of these calves developed body temperatures of 40°C (104°F). Two calves demonstrated neither a temperature elevation nor clinical signs of respiratory tract disease.

Serum samples were obtained from the calves before infection and 1 week, 1 month and 2 months after infection. The samples were tested for antibody against BRSV using a plaque reduction neutralization test. Four of the 5 calves demonstrated seroconversion (a four-fold or greater increase in serum antibody titer between pre-infection and post-infection samples) to the BRSV antigen. Two of the 5 calves failed to display evidence of neutralizing (maternally derived) antibody to BRSV in their sera at the time of experimental infection. These 2 calves also failed to display clinical signs of respiratory tract disease following inoculation. The researchers concluded that BRSV is capable of producing experimental illness in calves, particularly those possessing serum neutralizing antibody to BRSV at the time of infection.

The initial isolation of BRSV from cattle in Norway was also followed by an experimental study in a calf using the recovered isolate (Odegaard and Krogsrud, 1977). The animal developed mild clinical disease, virus was re-isolated from the upper respiratory tract, and production of antibodies was demonstrated.

In a rather complicated study, 3 different bovine isolates (Dorset, Hertfordshire and Swiss) of RSV were administered intranasally to conventionally-reared (n = 11), colostrum-deprived (n = 9) and gnotobiotic calves (n = 6) (Jacobs and Edington, 1975). Generally, a 10 milliliter (ml) volume of inoculum (third to fifth passage) was administered to each

calf. Calves ranged in age from 3 to 11 weeks at the time of experimental infection. Eighteen of the experimentally-infected calves were necropsied between 7 and 13 days post-inoculation. Results in 4 conventional calves were questionable due to the presence of bronchopneumonia in the experimental group prior to inoculation.

Clinical signs of respiratory tract disease were mild and included biphasic pyrexia, lethargy, nasal discharge and cough. Clinical signs were observed in 5 of 7 conventional calves, in only 1 of 9 colostrum-deprived calves and in 4 of 6 gnotobiotic calves. However, virus isolation from nasal secretions was successful 4 to 10 days after inoculation in the majority of experimental cases. Virus was also recovered from respiratory tract mucosa, tonsil, and lung in selected cases at necropsy.

Gross lesions were not observed at necropsy in any of the experimentally infected calves. Histologic lesions were reported in 12 animals and consisted primarily of a catarrhal bronchiolitis with the occasional formation of syncytia in bronchioles and alveoli.

Serum neutralization studies were used to determine BRSV antibody titers in calves both before and after experimental infection. Serology indicated that experimental infection is possible despite the presence of circulating antibodies. Also, passively-derived maternal antibody did not appear to cause disease exacerbation as previously reported (Smith et al., 1975).

The clinical signs and pathologic lesions produced in these experimental infections were considerably milder than the response commonly observed in actual field cases. The clinical response of susceptible calves did not vary significantly with changes in isolate, passage level, amount of virus inoculated or the cell system used to propagate the

virus. Researchers concluded that the virus may be only one of several factors necessary to experimentally reproduce the disease as it occurs naturally.

In an adjunct study, a gnotobiotic calf was inoculated with a human isolate of RSV recovered from an infant with acute bronchiolitis (Jacobs and Edington, 1975). The calf displayed a marked biphasic temperature response, but no other clinical abnormalities were noticed. Gross or histologic lesions were not present at necropsy. The investigators, however, concluded that HRSV is pathogenic for calves.

A report published early in 1975 represented the initial experimental attempt to conduct a controlled BRSV-infectivity study in calves (Mohanty et al., 1975). In this experiment, 11 conventionally-reared, 6 to 8 week old Holstein-Friesian bull calves were assigned to 4 experimental groups:

Group A (n = 3) - control calves.
Group B (n = 2) - one calf was inoculated with virus while the other served as a contact control.
Group C (n = 3) - all calves received viral inoculum.
Group D (n = 3) - all calves received viral inoculum.

Calves were inoculated with the Bovine X isolate (eleventh passage) of BRSV and 10 ml of inoculum ($10^{3.5}TCID_{50}$/ml) was administered intratracheally or aerosolized intranasally. Some calves received virus by both routes. Control calves were inoculated similarly with noninfected tissue culture fluid.

Calves were maintained up to 21 days post-inoculation. Selected control and principal calves were euthanatized and necropsied on days 14 and 21 of the study.

All calves treated with virus displayed a transient increase in body temperature (39.5°C/103.1°F - 40.5°C/104.9°F) on days 7 and

8. Mild cough, serous nasal discharge and slight hyperpnea were also occasionally observed. All control calves remained normal throughout the study.

Virus was successfully recovered from infected calves on days 7 and 9 and at necropsy. However, serologic response of these animals to the virus was extremely poor. Only low levels of neutralizing antibody were detected in the serum and nasal secretions.

At necropsy, gross lesions were minimal and consisted of small foci of pulmonary consolidation. Histopathologic changes indicative of interstitial pneumonia were present and included peribronchiolitis, squamous metaplasia of bronchiolar epithelium, mononuclear infiltration into alveolar walls and occasional infiltration of alveolar spaces with syncytial cells and neutrophils.

The authors of the previous report subsequently pursued an additional study in which calves received dual exposure to BRSV for the purpose of determining the role of serum and nasal neutralizing antibody on disease progression (Mohanty et al., 1976). Nine 6 to 8 week old bull calves were assigned to 4 experimental groups:

Group A (n = 3) - control calves.
Group B (n = 2) - all calves received virus.
Group C (n = 2) - all calves received virus.
Group D (n = 2) - one calf was inoculated with virus while the other served as a contact control.

The calves were inoculated both intranasally and intratracheally with 10 ml of the virus (titer = $10^{3.5}$ $TCID_{50}$/ml). BRSV isolate A51908, which had been passaged 5 times in tissue culture, was utilized in this study. During the initial challenge, 2 control calves were inoculated with noninfected tissue culture fluid while 2 others remained uninoculated.

An extremely mild clinical response followed viral inoculation. Most calves displayed a transient elevation in body temperature (39.5°C /103.1°F - 40.5°C/104.9°F), serous nasal discharge, mild cough or slight hyperpnea. Control calves (Group A) remained normal.

All virally-inoculated calves shed virus in nasal secretions for various lengths of time. The investigators discovered that virus isolation from nasal swabs was considerably more successful if material was inoculated into susceptible cell culture within 1 hour of collection.

Before inoculation, all calves possessed various titers (1:2 to 1:16) of serum neutralizing antibody to BRSV. Only 1 calf possessed detectable levels of neutralizing antibody in nasal secretions at this time. Following inoculation, infected calves responded serologically, but their titers of serum neutralizing (1:2 to 1:32) and nasal secretory antibody remained low.

Five weeks following initial exposure, all calves (Groups A, B, C, D) were challenged with the BRSV inoculum. Calves initially exposed to the virus (Groups B, C, D) were solidly immune after re-exposure and failed to display any signs of respiratory tract disease. The calves which served as controls during the initial exposure (Group A), however, displayed mild cough, hyperpnea and a transient elevation in body temperature when challenged. All previous control calves shed virus in their nasal secretions following viral challenge. Virus was isolated from nasal secretions in 3 of 5 calves after reexposure. Serologic response to the virus was poor in the previous controls. However, an appreciable antibody response was detected in serum (1:16 to 1:128) and nasal secretions (1:8 to 1:16) of calves challenged with the virus a second time. Two infected calves were euthanatized and necropsied on day

16 following rechallenge. Grossly, small areas of consolidation were evident. Histologically, pneumonia of variable severity was demonstrated in these regions.

It was concluded that disease due to BRSV occurs in the presence or absence of circulating serum antibodies. However, unlike previous reports originating from human medicine (Kim et al., 1969; Chanock et al., 1970) and veterinary medicine (Smith et al, 1975), there was no evidence that preexisting serum antibody caused exacerbation of the disease in young calves. Also, nasal secretory antibody appeared to protect calves against disease as calves previously exposed to the virus were immune to rechallenge.

To further define the role of BRSV infections in western Canadian cattle, experimental infectivity studies were conducted in 2 groups of calves by researchers at the University of Saskatchewan (Moteane et al., 1978). Five 1-week-old and six 7-month-old calves, determined to be free of neutralizing serum antibodies against BRSV, were infected intranasally with the Iowa isolate (FS1-1) of BRSV. Two milliliters of virus (titer = 10^5 PFU/ml) were aerosolized into the nasal passages of these animals.

Mild signs of respiratory disease were produced in four of five 1-week-old calves and in four of six 7-month-old calves. Observed signs included coughing, ocular discharge, mucopurulent nasal discharge, increased lung sounds and body temperatue elevation (39.4°C/103°F-39.7°C/103.5°F). Demonstrable changes in the hematocrit, white cell count, differential leukocyte count, plasma fibrinogen and total protein concentration were not observed. The virus was successfully reisolated from the nasal secretions of most calves. The 1-week-old

calves were euthanatized and necropsied at 3 day intervals following infection, but no gross or histologic changes were found. The extremely mild disease produced in these experimental studies actually prompted these investigators to question the importance of BRSV in the bovine respiratory disease complex.

Up to this point, all experimental BRSV studies in calves had resulted in mild clinical disease. English workers proposed that assessment of clinical response is particularly difficult when the ensuing disease is relatively mild. As a result, these investigators advocated adaptation of an unbiased and quantitated scoring system to use in conjunction with BRSV infectivity studies in calves (Thomas et al., 1977).

In this trial, a disease score was generated for each animal based on clinical (apathy, anorexia, nasal discharge, adenitis, ocular discharge, conjunctivitis, dyspnea, wasting, cough, diarrhea, respiratory rate and temperature) and hematological (RBC, WBC, MCV, PCV, hemoglobin) parameters evaluated during the experimental period. Resulting disease scores were contrasted between infected and control groups of calves.

Considerable detail was allotted to the experimental design of this study. However, despite careful planning, the value of this experimental study was diminished when the BRSV inoculum was discovered to be contaminated with BVD virus. Also, the proposed scoring system, although used occasionally (Stott et al., 1978; Elazhary et al., 1980), failed to gain wide acceptance by future investigators attempting experimental BRSV infections in calves.

In additional work originating from England, a significant association between RSV infection and natural outbreaks of respiratory disease was documented in young cattle on a large English beef farm (Stott et

al., 1978). This finding encouraged researchers to conduct further BRSV-inoculation studies in calves. An infectivity study was designed based upon the previously discussed disease-scoring system (Thomas et al., 1977). Clinical signs were graded and an illness score was calculated for each animal.

Respiratory syncytial virus was administered intranasally to 15 calves during two carefully controlled trials. Animals were successfully infected as judged by recovery of virus from nasal secretions and detection of a significant antibody response. However, there was no evidence that experimental RSV infection produced disease and mean illness scores did not differ significantly between infected animals and control calves.

Researchers judged that failure to experimentally produce disease with BRSV did not constitute strong evidence against this virus's causative role in the bovine respiratory disease complex. A pathogen may only produce disease under "appropriate conditions" and, until the pathogenesis of the disease is fully understood, the "appropriate conditions" may remain unknown.

The first infectivity study published in the '80s originated from Canada (Elazhary et al., 1980). Unlike previous efforts, this Canadian project reported moderate to severe signs of respiratory disease in the experimentally-infected animals. Disease scores, based on clinical and hematological findings, were assigned to experimental calves (Thomas et al., 1977). Final scores were considerably higher in infected calves than in controls.

Fourteen 1 to 5-week-old Holstein calves were utilized in this experiment. Eleven calves were exposed intranasally to the Quebec strain

of BRSV (titer = $2X10^6TCID_{50}$). Three calves were housed separately and served as controls. Controls received sterile tissue culture media inoculation.

Clinical response was classified as moderate in 2 of the infected calves and as severe in the other 9 animals. Pyrexia (maximum recorded temperature = $39.9^oC/103.8^oF$), leukopenia, nasal discharge, lung sounds, conjunctivitis and ocular discharge were the reported clinical and hematological observations. Clinical signs were most severe between days 6 to 9 post-infection. Inoculated calves shed BRSV in their nasal secretions from 1 to 12 days post-inoculation. Serologic response of infected calves was determined by the indirect fluorescent antibody technique. Two calves displayed antibody titers to BRSV of 1:128 by 9 days post-inoculation.

In another Canadian study, calves were inoculated with BRSV for the purpose of characterizing the interferon and serum-antibody response (Elazhary et al., 1981). Eleven 6-week-old Holstein calves, determined to be free of serum antibody to BRSV, were assigned to four groups:

Group I (n = 3) - inoculated once with virus.
Group II (n = 3) - rechallenged with virus on postinoculation day 15.
Group III (n = 3) - rechallenged with virus on postinoculation day 37.
Group IV (n = 2) - control group.

Experimental animals were inoculated with the Quebec strain of BRSV (titer = $2x10^5TCID_{50}$). Control calves were treated similarly with sterile tissue culture media.

Post-infection, all inoculated animals developed conjunctivitis, lacrimation, increased lung sounds, dyspnea and fever ($39^oC/102.2^oF$ to $41^oC/105.8^oF$). Clinical signs were most prominent on post-inoculation days 6 and 7, gradually disappearing by post-inoculation day 9.

Clinical signs of disease were not observed in Group II and Group III calves following reexposure to the virus. Control calves never displayed any signs of respiratory disease throughout the entire study.

Serum antibody response was monitored by the serum-neutralization and indirect fluorescent antibody tests. Antibody responses were similar in the three groups of infected calves and rechallenge with the virus in groups II and III did not stimulate an anamnestic response. The indirect fluorescent antibody test detected serum antibody in all calves as early as 3 days post-inoculation. Maximum titers appeared about 10 days post-inoculation and remained stable for at least 7-1/2 weeks before decreasing. The serum-neutralization technique also detected serum antibody in all calves by 3 days post-inoculation. However, serum-neutralization titers peaked at 4-5 weeks and then decreased. In general, higher titers were consistently obtained with the indirect fluorescent antibody test in contrast to the serum-neutralization procedure. BRSV antibodies were never detected in the sera of control calves.

A plaque-inhibition technique was used to assay serum interferon levels. Interferon titers peaked soon after viral inoculation in all calves, but then decreased to undetectable levels by 6 days post-inoculation. Moderate-to-low levels of interferon reappeared in most animals 1 week later and these titers persisted for a number of weeks. Interferon was never detected in the sera of control calves.

In an important experimental study, English workers established infections in conventional and gnotobiotic calves to develop and compare direct immunofluorescence with virus isolation as a diagnostic technique (Thomas and Stott, 1981). Fourteen conventionally-reared 5 to 9-month-old calves were inoculated intranasally with 5 ml of a RSV suspension

(1.9 plaque forming units x 10^5/ml). A total of 107 nasopharyngeal specimens were collected over a 30-day period following experimental infection. Twenty-four of the 107 specimens were positive by the direct fluorescent antibody method, while 21 of the 107 specimens proved positive by virus isolation. Virus isolation was most sensitive between 3 and 9 days post-inoculation, while immunofluorescence was most sensitive between 10 and 17 days post-infection. In a typical FA-positive nasopharyngeal prep, fluorescence was clearly confined to the cytoplasm of small, round (presumably phagocytic) cells. Nonspecific fluorescence associated with cellular debris occasionally made interpretation of some nasopharyngeal smears difficult.

Nine gnotobiotic calves were also experimentally infected with BRSV. These calves were euthanatized and lung sections were collected and stained by the direct immunofluorescent method. Fluorescent antigen was discovered in lung tissue from 8 of 9 gnotobiotic calves. Viral antigen was detectable in pulmonary sections up to 48 hours postmortem and appeared to be located in alveoli and bronchioles. Problems associated with non-specific fluorescence were not encountered during examination of lung tissue. Virus isolation was attempted on lung washings collected at necropsy and successful isolation occurred in two of nine samples.

Researchers concluded that direct immunofluorescence represented a reliable, quick and easy method of successfully diagnosing BRSV infection from nasopharyngeal and lung samples. The fluorescent antibody technique offers the advantage of speed and simplicity over both virus isolation and serology.

The most successful attempt to experimentally reproduce BRSV pneumonia in calves originated from Ireland in 1982 (Bryson et al., 1982;

Bryson et al., 1983; McNulty et al., 1983). In this thoughtfully conceived study, researchers modified the designs of previous investigators and thus were able to induce severe clinical disease and extensive pneumonia in their experimental subjects.

Seventeen calves (11 Friesian bulls, 5 Hereford/Friesian bulls, 1 Simmental heifer) were obtained from local dairy farms shortly after birth. All but 3 of the calves were colostrum-deprived. To prevent colisepticemia, bovine immunoglobulin-M was administered parenterally to colostrum-deprived calves upon arrival at the research facility. Calves were subsequently reared individually or in groups of 2.

A local strain of BRSV, isolated from the trachea of a dairy calf with pneumonia, was used to generate the inoculum required for this study. The inoculum contained between $10^{3.5}$ to $10^{6.5}$ $TCID_{50}$ of virus/ml. Calves were less than 7 days of age and free of respiratory tract illness at the time of inoculation. Calves were assigned to three experimental groups and inoculated according to the following design and schedule:

Group A (n = 9): Using a combined respiratory tract route, calves were inoculated twice daily for 4 consecutive days. The morning inoculation consisted of 10 ml of virus administered intranasally and 10 ml of virus administered intratracheally. In the afternoon, calves were given 10 ml of virus intranasally.

Group B (n = 3): These calves were inoculated intranasally with 10 ml of virus in the morning and again in the afternoon for 4 consecutive days.

Group C (n = 5): This group consisted of control animals inoculated with harvests from non-infected cell culture via the combined respiratory tract route for 4 consecutive days.

Post-inoculation, calves were examined daily and necessary samples were collected. To complete the study, infected calves were serially euthanatized and necropsied on post-inoculation days (PID) 1 to 13.

Respiratory signs were noted in all calves receiving the BRSV inoculum. The clinical picture, however, was considerably more severe in the group inoculated by the combined route than in calves inoculated by the intranasal route alone. Spontaneous coughing, tachypnea, marked hyperpnea, harsh lung sounds, respiratory distress, dyspnea, expiratory grunt and depression were frequently observed. Despite the assortment of severe clinical signs, 39.2°C/102.6°F was the highest body temperature recorded at any time during the study. Control calves remained clinically normal throughout the experiment.

Gross pneumonic lesions were induced in all calves inoculated with BRSV. Calves infected by the combined respiratory tract route displayed extensive and confluent areas of consolidation. The cranial, middle and cranioventral portions of the caudal lobes were primarily affected. In one Group A calf, severe septal edema and dissecting emphysematous bullae were present in the right caudal lobe. In calves inoculated solely by the intranasal route, gross lesions were minimal and consisted predominantly of scattered regions of lobular consolidation. Aside from small areas of superficial lobular collapse in 2 calves, no gross lesions were noted in control animals.

Histologically, the observed microscopic changes reflected a pathological continuum dependent upon the time interval between viral inoculation and necropsy examination. BRSV pneumonia had both exudative and proliferative components with the most severe damage observed in small bronchi, bronchioles and alveoli.

In Group A, calves necropsied on PID 1, 2 and 4 displayed varying degrees of bronchitis, bronchiolitis, cellular infiltrate into alveolar spaces and thickening of alveolar septa. Multinucleate epithelial syncytia, with eosinophilic intracytoplasmic inclusion bodies, were widespread. Re-epithelialization of damaged bronchiolar mucosa, organization of bronchiolar exudate, phagocytosis of cellular debris and peribronchiolar fibrosis was evident in the lungs of Group A calves necropsied on PID 10 and 13. Also, rare syncytia were occasionally detected in histologic sections from these animals.

In Group B calves, the predominant changes observed included bronchiolitis, alveolar cellular infiltrate and thickening of alveolar septa. No multinucleate epithelial syncytia were found in these animals. Notable histopathologic changes were not observed in control calves.

Meticulous efforts were undertaken to recover BRSV from experimentally-infected calves. RSV antigen was intermittently detected by immunofluorescence in nasopharyngeal cells present in samples of nasal mucus. Evidence of tracheal and pulmonary infection with RSV was obtained by immunofluorescence and virus isolation in calves inoculated by the combined route, but not in calves inoculated intranasally. Within the lung, RSV antigens were observed to fluoresce in bronchiolar and alveolar epithelium, in cellular debris located in airway lumens and occasionally within syncytial cells. No other respiratory viruses were demonstrated or isolated from infected calves. In controls, all upper and lower respiratory tract samples examined by immunofluorescence were negative for respiratory viruses. However, BVD was recovered from the lungs of 2 control calves using virus isolation procedures.

Evidence of serological conversion to RSV was obtained with infected, colostrum-deprived calves sampled late in the post-inoculation period (PID 10-13). Antibody titers were measured by the indirect immunofluorescent antibody technique. Calves which had received colostrum possessed demonstrable antibody titers to RSV prior to infection. Maternally deprived antibodies, however, did not protect calves from infection. Control calves displayed no detectable antibody response to BRSV, PI-3 or BVD, either before or after inoculation.

At necropsy, no bacterial isolations were made from the lower respiratory tract of any of the calves. A variety of non-pathogenic bacteria and fungi were recovered from nasopharyngeal swabs taken from both infected and control calves. A Mycoplasma isolate was reported from upper and lower respiratory tract samples originating from one experimentally-infected animal.

In conclusion, researchers demonstrated that severe respiratory disease could be induced experimentally in calves with BRSV. The successful results were attributed to the following factors:

1. BRSV isolate used to generate the inoculum
2. Infection by the combined respiratory route
3. Repeated inoculations
4. Younger age group of experimental animals
5. Season (majority of calves were inoculated during the autumn of the year, a time when many naturally occurring outbreaks of RSV pneumonia have been noted.)

The most recent experimental infectivity study was conducted in the United States and included an ultrastructural description of induced respiratory lesions, evaluation of BRSV's effect on alveolar macrophage function, and an attempt to identifiy adverse pulmonary sequelae arising from BRSV infection (Castleman et al., 1985a; Castleman et al., 1985b).

Colostrum-fed, male Holstein calves, obtained on the day of birth, were raised in an isolation facility. Calves were weaned at 30 days of

age and subsequently inoculated with BRSV one week after weaning. Twenty-one calves received solitary inoculation with virus (375 isolate) by combined intranasal aerosolization-intratracheal injection. Individual viral exposure varied from 10^4 to $10^{6.9}$ $TCID_{50}$. Six control calves were similarly inoculated with uninfected tissue culture media. Experimentally-infected calves were serially necropsied between post-inoculation days (PID) 2 and 30.

A mild clinical response ensued. Only 7 of 21 calves inoculated with BRSV developed signs of respiratory tract disease including serous nasal discharge, dry cough and elevated respiratory rate. At necropsy, macroscopic lesions were minimal with several calves possessing occasional red, firm lobules, irregularly distributed in the cranioventral lung field. Potential bacterial pathogens, including Corynebacterium pyogenes, Pasteurella sp and Mycoplasma sp, were periodically isolated from the lungs of both control and inoculated calves.

Bronchitis and tracheitis, characterized in part by epithelial necrosis, formation of syncytial epithelial cells and epithelial hyperplasia, were the predominant lesions observed histologically. Rhinitis, multifocal bronchiolitis and interstitial pneumonia were seen less frequently. Viral-induced lesions were resolved by 30 days post-inoculation. Measurable alterations in lung volume were not present 30 days after BRSV infection. Results of the present study suggested that BRSV inoculation of calves produced reversible modification of airway epithelial structure.

Ultrastructurally, viral nucleocapsids and budding virions were present in tracheal, bronchial and bronchiolar epithelial cells. Viral assembly in tracheal and bronchial epithelial cells was associated with

loss of cilia, formation of syncytial epithelial cells, swelling of mitochondria and endoplasmic reticulum and cell necrosis. Many epithelial cells contained intracytoplasmic nucleoprotein inclusions and intracytoplasmic remnants of the ciliary apparatus. Inflammatory cells were closely associated with virally infected and damaged epithelial cells. Swelling of type 1 and type 2 alveolar epithelial cells, interstitial edema and infiltration of inflammatory cells into alveolar septa indicated mild interstitial pneumonia. Investigators speculated that damaged mucociliary clearance could enhance susceptibility of inoculated calves to secondary bacterial infection.

Detection of virus in respiratory tract samples was accomplished by both virus isolation and immunofluorescent techniques. Fluorescent microscopy, however, proved to be the most sensitive indicator of viral antigen. Positive fluorescence was identified in alveolar macrophages and in the cytoplasm of nasal, pharyngeal, tracheal, bronchial, bronchiolar and cuboidal alveolar (type 2) epithelial cells. Viral antigen appeared most commonly in the cytoplasm of tracheal and bronchial epithelial cells 3 to 5 days after inoculation.

All experimentally-infected calves seroconverted to BRSV and increases in antibody titer were discovered as early as PID 3. The maximum titer reported in this study, as determined by serum neutralization, was 1:81. Passively-acquired maternal antibody to BRSV did not prevent viral infection or interfere with the seroconversion response.

Alveolar macrophages were recovered by bronchoalveolar lavage of the left caudal lung lobe. Phagocytosis was evaluated by quantitating the rate at which 2.02 um polyvinyl toluene beads were uptaken by these

cells. Alveolar macrophages exposed to BRSV in vivo demonstrated increases in phagocytic rate at 3, 5 and 7 days post-inoculation. The elevation observed at 5 days post-inoculation was determined to be statistically significant ($p < 0.01$).

BRSV inoculation produced a subdued clinical response with minimal pulmonary pathology in this study. Overall, the observed responses following experimental infection have been highly variable. These authors offered several suggestions to explain the variability associated with experimental BRSV infections in calves:

1. inherent pathogenicity and cellular tropism of the viral isolate.
2. attenuation of viral pathogenicity following repeated tissue culture passage.
3. concurrent pulmonary bacterial and viral infection.
4. host factors (i.e. variation in the intensity of pulmonary cell injury associated with the immune response directed against virally-infected cells).

II. Experimental BRSV Infections in Gnotobiotic Calves

The work of Bryson (1982,83), McNulty (1983) and several earlier investigators indicated that BRSV may be an important pathogen of the bovine respiratory tract. To further substantiate this belief, infectivity studies were undertaken in gnotobiotic calves (Thomas et al., 1984a). By inoculating calves raised and maintained in strict microbiological isolation, all induced clinical disease and pulmonary pathology could confidently be associated with BRSV infection.

Eight Friesian or Angus/Friesian gnotobiotic calves, 1-3 months of age, were inoculated intranasally with the Snook isolate (eighth passage) of BRSV. Calves received between 3,000 and 50,000 plaque forming units of virus in 10 ml of culture fluid inoculum. All inoculations, samplings or examination procedures were conducted within the gnotobiotic environment.

None of the 8 calves developed recognizable clinical signs of respiratory tract disease. However, at necropsy, 7-14 days following inoculation, moderate to substantial pneumonic lesions were observed in all 8 calves. Lung lesions were recorded on a standard lung diagram and a percentage of the affected surface area was estimated. Two to twenty-five percent consolidation was reported. Despite gnotobiotic conditions, none of the lungs were found to bacteriologically sterile at the conclusion of the experiments. None of the isolated bacterial species, however, were considered significant pathogens in the bovine respiratory disease complex. Histologic lesions consisted of exudative and proliferative bronchiolitis with accompanying alveolar collapse, thickening of alveolar walls, alveolar epithelialization and accumulation of alveolar exudate. Multinucleate epithelial syncytia were not predominant microscopic findings.

Recovery or detection of viral antigen appeared to be dependent upon the time of sampling after inoculation. Virus was recovered from the nasopharynx between 2 and 11 days post-inoculation and from the lung up to and including day 9 following inoculation. Antigen was detected by immunofluorescence in nasopharyngeal smears, nasal mucosa, tracheal mucosa and lung samples. Fluorescent antigen was commonly observed in bronchiolar epithelial cells and in detached epithelial cells comprising bronchiolar and alveolar exudates.

Serum antibody to BRSV was not present in pre-inoculation samples. A serological response to BRSV, however, was demonstrated by virus neutralization and single radial hemolysis at 11 days post-inoculation. Radioimmunoassay was utilized to measure class-specific antibodies to RSV and IgM was first detected as early as 9 days post-infection.

Researchers felt that demonstration of BRSV-induced pathology in the lungs of gnotobiotic calves represented an important advance. They also suggested that the close resemblance between the experimental disease in calves and the pathology of acute bronchiolitis in children qualify cattle as a particularly relevant model for the human disease.

A subsequent study involving gnotobiotic calves ensued (Thomas et al., 1984b). In this effort, bovine and human strains of RSV were administered to gnotobiotic animals. Additionally, the influence of stress on disease progression, in the form of dexamethasone treatment, was also investigated.

Thirteen calves were inoculated with the Compton 127 isolate of BRSV (twenty-third passage) while 7 calves received the Human A2 isolate of HRSV (this strain had been subjected to numerous passages in both humans and bovine cell culture prior to inoculation). Three different inoculation procedures were utilized: intranasal, combined intranasal and

intratracheal administration, and aerosol spray. Four calves, inoculated with the bovine strain by the combined respiratory route, were also treated with dexamethasone (0.5 mg/kg body weight, IM, once per day) for a total of 10 days.

Both viral strains proved capable of infecting calves, but neither strain induced clinical signs of respiratory tract disease. In dexamethasone-treated calves, the authors reported the appearance of clinical signs. However, none of the described clinical signs (alopecia, apathy, scleral injection) were particularly specific for respiratory tract disease.

At necropsy, no significant gross lesions were observed in the lungs of inoculated calves. Histologically, round mononuclear cells were observed infiltrating into peribronchiolar tissue and alveolar walls. In dexamethasone-treated animals, small areas of pneumonic consolidation were noted in 3 of the 4 calves (2%, 4%, 6% of pulmonary surface area). In addition to the previously described microscopic changes, mild exudative bronchiolitis and alveolitis were also detected in these animals.

Both strains of virus were successfully isolated from the nasopharynx of inoculated calves. Peak recoveries occurred between 3 and 8 days following inoculation. Dexamethasone appeared to prolong the period of viral shedding and to increase the harvest of isolated virus. Immunofluorescence detected viral antigen in only 4 of 13 lungs examined.

The serological response to both strains was similar. Antibody to RSV was not found in pre-inoculation serum samples, but was detected by virus neutralization or single radial hemolysis at about 10-12 days post-inoculation. Radioimmunoassay detected specific anti-RSV IgM at 10 days post-inoculation and specific anti-RSV IgG at 16 days post-inoculation.

Experimental infection of gnotobiotic calves with RSV produced an extremely mild clinical and pathological response in this study. The authors attributed these results to the passage history of the viral strains utilized in the experiment. In conclusion, this study indicated that human and bovine strains of RSV possess similar growth and biological characteristics in gnotobiotic calves. Dexamethasone treatment extended the period of viral shedding, enhanced the amount of virus recovered and slightly increased the severity of observed lesions.

III. Experimental BRSV Infections in Sheep

Respiratory disease accounts for significant financial losses in the sheep industry. Although naturally occurring RSV infections in sheep have not been well characterized or studied, the discovery of specific antibodies to BRSV in sheep serum (Berthiaume et al., 1973; Smith et al., 1975) inspired researchers to undertake infectivity experiments in this species (Lehmkuhl and Cutlip, 1979a; Cutlip and Lehmkuhl, 1979). In the first of these studies, 5 colostrum-deprived lambs, approximately 1 week old, were inoculated intranasally and intratracheally with 20 ml of the 375 isolate (third passage) of BRSV. The inoculum contained $10^{3.6}$ plaque forming units/ml. Lambs were observed daily for development of respiratory tract disease. Necropsies were conducted between post-inoculation days 7 and 28.

Three of the lambs responded with fever (maximum recorded temperature - 40.8°C/105.4°F), hyperpnea and depression. Grossly, multiple hemorrhagic and consolidated foci were observed throughout all lung lobes. Histologically, the basic lesion was a focal interstitial pneumonia. Microscopic features included macrophage infiltration into

alveolar septa and alveolar sacs, epithelial necrosis and accumulation of cellular debris in airways and alveoli. Epithelial hyperplasia was detected in many alveoli, but multinucleated epithelial syncytia were rare. Affected foci were surrounded by relatively large zones of normal pulmonary tissue. Similar changes were observed with electron microscopy. In addition, pleomorphic virions were seen budding from airway and alveolar epithelial cells.

Virus was isolated from the nasal secretions, tracheal fluids and pulmonary tissue of 2 lambs. Results of an agar gel immunodiffusion test indicated that the recovered virus was identical to the strain present in the inoculum. None of the experimental lambs possessed pre-inoculation serum antibody titers to BRSV. Post-inoculation, all lambs seroconverted to the viral antigen.

Results of this study indicated that BRSV is capable of infecting and producing disease in sheep. The mild clinical response induced in lambs was similar to several of the experimental results described in calves.

Lehmkuhl and Cutlip (1979b) subsequently conducted a similar experimental study involving 6-month-old (feedlot age) lambs. Six colostrum-deprived lambs, raised in isolation, were inoculated intratracheally with 20 ml of the 232 isolate of BRSV (this isolate was recovered from the lung of a lamb originally inoculated with a bovine strain of RSV). The inoculum contained $10^{3.9}$ plaque forming units/ml. Lambs were observed daily for respiratory tract disease. Serial necropsies were conducted between post-inoculation days 3 and 30.

Five of the lambs responded with hyperpnea, lethargy and transient pyrexia (maximum recorded temperature 40.8°C/105.4°F). Grossly, lung

tissue appeared normal except for petechiae in the cranial and intermediate lobes. Microscopically, lesions were present in all lobes and consisted primarily of multifocal areas of interstitial pneumonia and bronchiolitis. Virus was recovered from the nasal secretions, tracheal fluids and pulmonary tissue of 5 lambs. All lambs possessed serum antibody to BRSV prior to inoculation. Post-inoculation, large increases in antibody titers were detected by the plaque-reduction test.

Based on the mild clinical response produced by experimental inoculation, researchers inferred that severe BRSV pneumonia must originate from a complex, multifactorial pathogenesis. The lesions produced by BRSV may enable pathogenic or opportunistic bacteria to colonize the lung and contribute to severe respiratory disease.

Researchers from Washington attempted to objectively evaluate BRSV's potential to produce pneumonia in sheep by carefully simulating natural exposure under experimental conditions (Trigo et al., 1984a). Forty, conventionally reared, 6-month-old, Suffolk-cross sheep, seronegative to BRSV, were obtained for this study. Lambs were assigned to 2 experimental groups and were housed in closed, self-contained pens with filtered air systems and regulated temperatures. Inoculum was aerosolized via the intranasal route. Thirty animals in Group A received 20 ml of virus (strain ATCC VR-794; titer = $10^7 TCID_{50}$/ml) while 10 control lambs in Group B received 20 ml of tissue culture fluid. Clinical evaluations were recorded daily. One control and 3 inoculated lambs were subsequently euthanatized and necropsied on post-inoculation days (PID) 2, 4, 6, 9, 12, 15, 18, 22, 30 and 40.

Mild respiratory tract disease ensued. Inoculated lambs displayed transient fever, elevated respiratory rates and serous oculonasal

discharges. Gross lesions were minimal. Two lambs exhibited multifocal areas of consolidation in the cranial lobes. Histologically, bronchiolitis and alveolitis were present in numerous lambs. No multinucleated syncytial cells were detected. Ultrastructurally, bronchiolar epithelium appeared well-preserved. Occasional necrosis of type I and type II alveolar epithelial cells was noticed. Viral particles could be seen budding from the cellular membrane of type I pneumocytes.

Isolation of BRSV from nasal swabs or pulmonary tissue was possible only with samples collected within 6 days after inoculation. Specific immunofluorescence was detected in alveolar walls, alveolar macrophages and bronchiolar epithelium. Serologically, all but 2 inoculated lambs developed detectable antibodies to BRSV by PID 11. Serum titers peaked between PID 13 to 15. Neutralizing antibodies were also detected in pulmonary washings, but not in nasal secretions. Bacterial isolation from nasal swabs and pulmonary tissue produced a variety of microorganisms.

The observed experimental results demonstrated BRSV's potential to produce pneumonia in sheep under natural conditions. The authors suggested that interspecies transmission of BRSV between calves and sheep should be considered when these species are managed together.

IV. BRSV and *Pasteurella haemolytica* Syngerism in Sheep

Respiratory tract infections with BRSV have been successfully induced in sheep under experimental conditions (Lehmkuhl and Cutlip, 1979a,b; Cutlip and Lehmkuhl, 1979; Trigo *et al.*, 1984a). The resultant clinical and pathological response was considerably milder than the severe disease commonly associated with natural outbreaks of sheep

pneumonia. However, BRSV infection may predispose the ovine lung to the deleterious effects of secondary bacterial invaders (Lehmkuhl and Cutlip, 1979a,b; Cutlip and Lehmkuhl, 1979). Therefore, a well-designed experiment was undertaken in sheep to investigate the possible synergistic relationship between BRSV and Pasteurella haemolytica (Al-Darraji et al., 1982a,b,c).

Twenty-one colostrum-deprived 4-week-old lambs were assigned to five experimental groups:

Group 1 (n = 4)	Pasteurella haemolytica only.
Group 2 (n = 4)	BRSV only.
Group 3 (n = 5)	BRSV + P. haemolytica with a 3 day interval between viral and bacterial inoculation.
Group 4 (n = 6)	BRSV + P. haemolytica with a 5 day interval between inoculations.
Group 5 (n = 2)	control

All inoculations were administered intratracheally. BRSV isolate 232 was used to propagate the viral inoculum. Lambs received 20 ml of cell culture fluid containing 2.9×10^4 plaque-forming units of virus/ml. Pasteurella haemolytica biotype A serotype 1 was used for production of the bacterial inoculum. Lambs were given 5 ml of tryptose broth containing a viable cell count of 3×10^7 colony-forming units/ml. Following inoculation, lambs were observed twice daily for clinical signs of disease. Lambs were subsequently euthanatized and necropsied between 2 and 9 days post-inoculation.

Lambs inoculated with P. haemolytica (Group 1) or BRSV (Group 2) developed a mild respiratory tract disease accompanied by transient pyrexia, lethargy, dyspnea and hyperpnea in some animals. Although mild, the clinical signs seen in lambs given BRSV alone were more pronounced than signs in lambs given P. haemolytica alone.

Combined BRSV and P. haemolytica administration (Groups 3 and 4) produced a more profound clinical response than did either agent alone. Respiratory signs were more severe, more persistent, involved a higher percentage of lambs and included pyrexia, lethargy, nasal discharge, hyperpnea, dyspnea, coughing, anorexia, loss of condition and recumbency. Clinical signs were more severe in lambs inoculated with P. haemolytica 5 days after RSV (Group 4) than in those lambs inoculated with P. haemolytica 3 days after RSV (Group 3).

Pneumonic lesions were considerably more severe in lambs inoculated with BRSV and P. haemolytica than in lambs inoculated with either agent alone. In Group 1 (P. haemolytica only), lesions developed in 2 of 4 inoculated lambs. Grossly, fibrinous pleuritis with areas of pulmonary consolidation, necrosis and hemorrhage were observed. Macroscopic lesions were estimated to involve approximately 14% of the lung surface. Histologically, observed lesions were classified as acute fibrinopurulent bronchopneumonia with coagulative necrosis and fibrinous pleuritis. Concentric zonal lesions were encountered periodically. In Group 2 (BRSV only), 3 of 4 inoculated lambs displayed pulmonary lesions. Grossly, multifocal areas of consolidation and hemorrhage were scattered throughout the lung. Gross pathology involved approximately 5% of the pulmonary surface. Microscopic findings included bronchitis, bronchiolitis, peribronchiolitis and interstitial pneumonitis. Multinucleated giant cells were occasionally detected within the alveolar spaces. In Groups 3 and 4 (BRSV followed by P. haemolytica), extensive lesions, involving over 15% to 21% of the pulmonary surface, were present in 11 of 11 inoculated lambs. Pathology possessed characteristics of both bacterial and viral infections. Gross features included consolidation, abscessation,

necrosis, hemorrhage, emphysema and atelectasis. Fibrinous pleuritis, hydropericardium and pleural effusion were also commonly encountered. Microscopic pathology consisted of a severe exudative pneumonia and bronchiolitis with interstitial pneumonitis, focal necrosis and hemorrhage. Multinucleated giant cells and concentric zonal lesions represented additional histologic findings in these lambs.

Ultrastructural lesions were more prominent in lambs receiving both BRSV and P. haemolytica than in those lambs inoculated with either agent individually. Similar cytopathologic changes were found in lambs inoculated with P. haemolytica or with BRSV and P. haemolytica. In consolidated regions of lung, alveolar and bronchiolar lumens were filled with neutrophils, macrophages, erythrocytes and necrotic cellular debris. There were increasing numbers of type II pneumocytes. Bacteria were seen infrequently. In lambs inoculated with BRSV or with BRSV and P. haemolytica, viral buds were present on the plasma membranes of bronchial and bronchiolar epithelial cells. Individual epithelial cells in the smaller airways appeared necrotic. Multinucleated epithelial cells were occasionally observed.

Abundant viral antigen was detected by immunofluorescence in the lungs of lambs inoculated with BRSV or with BRSV and P. haemolytica. Positive fluorescence was observed in bronchial and bronchiolar epithelium, within the cytoplasm of cells located in the alveolar and bronchiolar lumens and, to a lesser extent, within surface epithelial cells of the nasal turbinates and trachea. Viral antigen was not detected in alveolar epithelium. Immunofluorescence was most intense in pulmonary samples obtained from lambs euthanatized earliest in the course of the experiment. Maximal viral replication and intracellular antigen accumulation apparently occurs soon after viral infection.

In conclusion, infection of colostrum-deprived lambs with BRSV and Pasteurella haemolytica resulted in severe, acute respiratory disease. The clinical and pathological severity reflects a synergistic relationship between these agents. The exact mechanism by which BRSV potentiates P. haemolytica infection in the lungs of lambs, however, remains speculative. Destruction of bronchial and bronchiolar epithelium by BRSV probably permits Pasteurella infections to become firmly established within the lungs. Diminution of pulmonary defense mechanisms by BRSV infection may also serve to enhance the spread of Pasteurella throughout the pulmonary parenchyma.

Although the previous study demonstrated severe pneumonia in colostrum-deprived lambs following intratracheal administration of BRSV and P. haemolytica (Al-Darraji et al., 1982a,b,c), researchers from the state of Washington challenged the relevance of the experimental design and therefore questioned the value of the derived results. Intratracheal administration of respiratory tract pathogens to colostrum deprived animals reflects little of what occurs under natural conditions. For these reasons, a similar study was conducted in which investigators attempted to carefully pattern experimental conditions after naturally-occurring disease situations (Trigo et al., 1984b).

Thirty-eight conventionally-reared, 3-month-old Finn sheep, seronegative to BRSV, were secured for this study. Lambs were housed in closed, self-contained pens with filtered air systems and regulated temperature. Lambs were randomly assigned to seven different experimental groups. Inoculation protocol varied among groups:

A (n = 5) control
B (n = 5) BRSV only
C (n = 6) P. haemolytica only

D (n = 6) BRSV + P. haemolytica on PID 3
E (n = 6) BRSV + P. haemolytica on PID 6
F (n = 5) P. haemolytica + BRSV on PID 3
G (n = 5) BRSV + P. haemolytica, simultaneous administration

Lambs were inoculated by a combined aerosol/intranasal exposure. Twenty milliliters of viral (isolate ATCC VR-794; titer = $10^6 TCID_{50}$/ml) and/or 20 ml of bacterial (P. haemolytica biotype A, serotype 1, ovine origin; titer = $8x10^8$ colony-forming units/ml) inocula were administered. Clinical evaluations were recorded daily. Lambs were subsequently euthanatized between 3 and 12 days post-inoculation.

Mild respiratory tract disease (pyrexia, oculonasal discharge) was observed in all the inoculated groups. Additional clinical signs (depression, elevated respiratory rate) were noticed in lambs from Groups D and E (BRSV + P. haemolytica).

Gross pulmonary lesions were observed only in groups exposed to both BRSV and P. haemolytica (3 of 6 lambs in Group D, 3 of 6 lambs in Group E, 1 of 5 lambs in Group G). Macroscopic lesions consisted of firm, red, well-demarcated regions of consolidation with a cranioventral distribution. Gross pathology was estimated to involve between 5% to 15% of the pulmonary surface. Histologically, the predominant lesion was a suppurative bronchopneumonia.

BRSV and P. haemolytica were successfully recovered from nasal secretions and pulmonary tissue of inoculated lambs. Immunofluorescence studies demonstrated concentration of viral antigen within alveolar cells, some alveolar macrophages, and a few bronchiolar epithelial cells.

In most lambs, seroconversion to the virus was not detected by the serum-neutralization procedure because lambs were euthanatized before peak antibody titers could develop. Of those groups receiving bacterial inoculum, many lambs seroconverted to P. haemolytica. Antibodies against

P. haemolytica were measured with the indirect hemagglutination test. Low concentrations of antibodies to BRSV and P. haemolytica were also detected in some lung lavage samples collected at postmortem. These low titers of antibodies were present in both control and inoculated lambs, indicating a possible explanation as to why all lambs receiving the virus-bacteria treatment did not develop disease. The presence of neutralizing antibodies in pulmonary secretions could inactivate the viral and/or bacterial inoculum.

Alveolar macrophages were also recovered from lung lavage fluid collected at necropsy. Latex phagocytosis and Fc receptor assays were conducted to evaluate alveolar macrophage function. Decreased Fc receptors and latex phagocytosis were detected in alveolar macrophages originating from Group E lambs (BRSV + P. haemolytica on PID 6).

Seemingly, BRSV does facilitate P. haemolytica pulmonary infection in conventional, immunocompetent lambs. Respiratory tract viruses may predispose lambs to bacterial pneumonia by compromising pulmonary antibacterial defenses. Depressed alveolar macrophage function was documented in this study.

LITERATURE REVIEW - Summary and Conclusions

Numerous attempts at reproducing BRSV pneumonia have been conducted in experimental calves and lambs during the past 10 to 15 years. The majority of experimental efforts have recreated mild clinical disease with minimal pulmonary lesions (Inaba et al, 1972; Smith et al., 1975; Mohanty et al., 1975; Moteane et al., 1978; Castleman et al., 1985a,b). Bryson (1982, 83) and McNulty (1983) had the greatest success at inducing severe pneumonia and pathology in calves with BRSV. Before many of the experimental studies are judged as failures, it is important to realize that disease indices associated with natural BRSV outbreaks also vary in intensity from mild to severe. Severe experimental disease probably represents an appropriate integration of numerous variables including host factors, experimental design, pathogenicity and passage level of viral isolate, and concurrent pulmonary bacterial and viral infections.

Calves are currently considered to be the most relevant animal model for study of RSV infection (Thomas et al., 1984a; Stott and Taylor, 1985). Newborn bull calves represent an inexpensive and readily available source of experimental animals. Calves, along with humans, are both natural hosts for RSV infections. Neutralization tests have demonstrated antigenic similarities between HRSV and BRSV (Inaba et al., 1972: Smith et al., 1975). Histological lesions and distribution of viral antigen in experimentally-infected calves closely resemble the pattern seen in infants with fatal RSV disease (Thomas and Stott, 1981; Bryson et al., 1982, 83; McNulty et al., 1983; Thomas et al., 1984a; Castleman et al., 1985a). Finally, HRSV is capable of infecting calves under experimental conditions (Jacobs and Edington, 1985; Thomas et al., 1984b).

OBJECTIVES

The majority of experimental attempts to reproduce repiratory tract disease with BRSV in calves have only resulted in mild clinical signs with limited respiratory tract pathology. A recent investigation (Bryson et al., 1982, 83; McNulty et al., 1983) was considerably more successful at inducing severe disease and pathology in calves. However, the experimental animals used in this study consisted of colostrum-deprived calves less than 7 days of age at the time of inoculation.

Most of these studies have failed to consider epidemiologic observations made from numerous field outbreaks in which BRSV pneumonia was described as being a biphasic respiratory tract disease (Wellemans et al., 1978; Bohlender et al., 1982; Frey, 1982; Baker et al., 1986b). In these reports, BRSV infections resulted in two recognizable stages of disease. An initial stage, featuring mild respiratory tract signs, was followed by a second stage, which featured dyspnea and severe respiratory distress. As a result of these observations, BRSV pneumonia has been hypothesized, but not proven, to be attributable to a hypersensitivity reaction or to the development of a persistent infection culminating in a hypersensitivity reaction (Bohlender et al., 1982; Frey, 1982; Frey, 1983). If the disease truly is a manifestation of a hypersensitivity reaction, it may be necessary to either rechallenge calves with BRSV to reproduce the disease or to maintain calves a longer period of time post-infection to determine if a persistent infection (known to occur with paramyxoviruses) culminating in a hypersensitivity reaction actually ensues.

In the present research, BRSV was administered to colostrum-fed dairy calves in accordance with the protocol of Bryson (1982, 83). All calves ranged between 3.5 and 4.5 weeks of age at the time of experimental infection. Objectives of this study were as follows:

1. To attempt to experimentally reproduce severe respiratory tract disease with BRSV in 1-month-old, colostrum-fed calves.
2. To determine whether single exposure to BRSV is capable of inducing severe respiratory tract disease or if a reexposure to the virus is necessary.
3. To investigate the hypothesized role of persistent infection and viral hypersensitivity in the pathogenesis of BRSV pneumonia.

Four groups of experimental animals were investigated during this study. Group I consisted of six control calves. Group II calves were inoculated with BRSV and subsequently necropsied during maximal clinical response. Group III was composed of six calves inoculated with BRSV, observed for 21 days, and then necropsied. These calves were observed for the development of biphasic respiratory tract disease or for the development of a persistent infection culminating in a viral hypersensitivity reaction. Group IV calves received a dual exposure to the BRSV inoculum. This group was included to determine if BRSV rechallange resulted in a hypersensitivity reaction or if reexposure to the virus was necessary to produce severe respiratory tract disease.

MATERIALS AND METHODS

1. Experimental Design

Four groups of calves were evaluated during this study. Each group consisted of six replicates. Experimental groups were investigated independently, but in succession, of each other. All experimentation was conducted during the spring and early summer. A summary of the four groups follows:

- Group I: Six control calves were sham inoculated, maintained for 21 days, and then necropsied.
- Group II: Six calves were inoculated with BRSV and subsequently necropsied during maximal clinical response (4-6 days post-inoculation).
- Group III: Six calves were inoculated with BRSV, observed for 21 days, and then necropsied.
- Group IV: Six calves received a dual exposure to the BRSV inoculum. A 10-day time period intervened between the two inoculations. The calves were observed for 21 days total, and then necropsied.

2. Experimental Animals and Housing

Holstein male calves, ranging in age from 2-6 days, were obtained from a local 1500-cow dairy. All calves received colostrum at birth. Following a cursory physical examination on the farm, calves selected as experimental subjects were transported to an isolation facility located on the Veterinary Research Farm at Michigan State University. In preparation for the calves' arrival, isolation rooms and husbandry equipment were chemically disinfected. Upon arrival at the isolation compound, experimental animals received 500,000 IU of vitamin A, 75,000 IU of vitamin D_3 (Vitamin A & D Injectable Solution, Pfizer, Inc.,

New York, NY), 200 IU of d-alpha tocopheryl acetate, and 3 mg of selenium (Bo-Se, Schering Corp., Kenilworth, NJ) intramuscularly. Experimental subjects were also maintained on systemic antibiotics (Procaine Penicillin G, Pfizer, Inc., New York, NY; Gentocin Solution, Schering Corp., Kenilworth, NJ) for approximately one week following the stress of transport to prevent the occurrence of bacterial pneumonia. Antibiotic administration was discontinued well in advance of viral inoculation.

At the isolation barn, 2-3 calves were assigned to an isolation room. Calves were reared individually in elevated metal stalls. To minimize introduction of infectious agents, operators wore surgical scrub suits, disposable paper coveralls, surgical caps, masks, gloves, and disposable polyethylene footwear when handling, feeding, examining, or sampling experimental animals. Calves were fed a high quality, non-medicated milk replacer (Fresh Start, Vita Plus Corp., Madison, WI). Each calf received a quantity of reconstituted milk replacer equivalent to 10% of its body weight, divided into 2 equal feedings per day, for the duration of the experiment.

3. Carotid Artery Relocation - Surgical Technique

Surgery was performed on each calf to exteriorize and isolate the left common carotid artery in a subcutaneous location in the mid-cervical region. All surgeries were conducted at the isolation facility. Anesthesia was produced by parenteral administeration of xylazine (Rompun, Mobay Corp., Shawnee, KA) and ketamine (Vetalar, Parke-Davis, Morris Plains, NJ) (Waterman, 1981). A 10 cm skin incision was made parallel and just ventral to the jugular vein in the middle third of the neck. The carotid artery was exposed by sharp and blunt dissection through the sternomandibular and sternomastoid muscles. A 5 cm length of carotid

artery was dissected free from the surrounding tissues, elevated, and the underlying muscle bellies were apposed with absorbable sutures (2-0 Chromic Gut, Ethicon, Inc., Somerville, NJ) in an interrupted pattern. The skin was closed with nylon (2-0 Ethilon, Ethicon, Inc., Somerville, NJ) in an interrupted pattern. The calves were allowed an additional one-week recovery period following surgery prior to viral inoculation.

4. Preparation of BRSV Inoculum

A. Isolate

The isolate of BRSV utilized in this study was recovered during an outbreak of calf pneumonia in Minnesota. The virus was propagated by four passages through bovine turbinate cell culture. One and one-half milliter aliquots of virus at the fourth passage level were stored at -70°C until needed.

B. Cell Culture

A bovine turbinate cell line was obtained from the National Veterinary Service Laboratory, Ames, IA. Cell cultures were maintained at 37°C in Eagle's minimal essential medium (KC Biological, Lenexa, KA) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), sodium bicarbonate (2.2 mg/ml) (Fisher Scientific, Fair Lawn, NJ), gentamicin sulfate (25 ug/ml) (Gentamicin Reagent Solution, Grand Island Biological, Grand Island, NY), sodium penicillin G (50 ug/ml), streptomycin sulfate (50 ug/ml), neomycin sulfate (100 ug/ml) (PSN Antibiotic Mixture, Grand Island Biological, Grand Island, NY), and amphotericin B (2.5 ug/ml) (Squibb and Sons, New Brunswick, NJ). Adventitious viral contaminants in fetal bovine serum were chemically inactivated with binary ethyleneimine (Bahnemann, 1976). Cell cultures were demonstrated to be free of noncytopathic bovine viral diarrhea virus (BVDV) by direct immunofluorescence testing.

C. Bacterial and Viral Screening of Stock Virus

Stock virus was screened for the presence of bacterial and viral contaminants. Stock aliquots of fourth-passage virus were cultured by the Animal Health Diagnostic Laboratory at Michigan State University and were determined to be free of aerobic bacteria, mycoplasmal and ureaplasmal agents. In addition, the stock virus was also screened for contamination with noncytopathic BVDV by subcutaneous administration to a colostrum-deprived calf and subsequent evaluation of the calf's serologic response. To accomplish this, a bull calf was immediately removed from its dam following birth, and was transferred to the isolation facility. Serum samples were obtained from the calf at birth, and at 48 hours of age, and serology for BVDV was performed by the Animal Health Diagnostic Laboratory at Michigan State University. No neutralizing antibodies to BVDV were detected in these initial serum samples (BVDV-VN titer < 1:4). Beginning at 2 days of age, the calf was inoculated subcutaneously with one ml of fourth-passage BRSV once a day for 3 consecutive days. If the stock solutions were contaminated with noncytopathic BVDV, seroconversion to BVDV would be expected in the colostrum-deprived calf. Serum samples were obtained at 8, 14, and 21 days post-inoculation. No neutralizing antibodies to BVDV were detected in these samples (BVDV-VN titer < 1:4). To confirm immunocompetence to BVDV in this animal, a modified live bovine rhinotracheitis-virus diarrhea-parainfluenza-3 virus vaccine (Resbo 3, Norden Laboratories, Inc., Lincoln, NB) was administered intramuscularly to this animal on 3 consecutive days. Positive seroconversion to BVDV was detected in serum samples collected 10 (BVDV-VN titer 1:128) and 17 days (BVDV-VN titer 1:512) post-vaccination.

D. Propagation of Virus

The BRSV was propagated in bovine turbinate cell culture as inocula was needed for experimental infections. Composition of cell culture media was similar as previously described, except that 10% horse serum replaced 10% fetal bovine serum. Tissue culture flasks (150 cm^2) (Corning Glassworks, Corning, NY) were inoculated with 1.5 ml of fourth-passage virus when the bovine turbinate cells had reached approximately 90% confluency. Viral adsorption was allowed to occur for one hour at room temperature. The media was changed, and the flasks were incubated at 37°C. When 20-25% of the cell monolayer displayed evidence of virus-induced cytopathic effect (virus-induced cytopathic effect or syncytial cell formation occurred 72-96 hours post-inoculation), virus was harvested. The growth media was decanted and replaced with approximately 11 ml of a 44% sucrose-20% horse serum media. The decanted growth media served as an intranasal inoculum for the experimental infections. The cell cultures were then frozen at -70°C and thawed for five cycles to promote rupture of intact cells and release of virus into the sucrose media. Sucrose is believed to have a protective effect on BRSV viability (Frey, M. Personal Communication, 1982). The sucrose media served as an intratracheal inoculum for the experimental infections.

E. Titration of Virus

Stock virus and viral inoculum were titered using the methods described by Carbrey et al. (1972), and the 50% endpoint of the virus titer was calculated by the method of Karber (1931). Fourth-passage stock virus contained between $10^{5.8}$ and $10^{5.9}$ $TCID_{50}$ of virus/ml. The intranasal inoculum (decanted growth media) contained between $10^{4.8}$

and $10^{4.9}$ $TCID_{50}$ of virus/ml. The titer of the intratracheal inoculum (sucrose-horse serum media) was between $10^{6.2}$ and $10^{6.3}$ $TCID_{50}$ of virus/ml.

5. Inoculation of Calves

Any calf displaying evidence of respiratory tract disease at the time of inoculation was disqualified from the study. Extra calves had been processed with each experimental group in anticipation of this possibility. The inoculation protocol utilized in this study was based on the methods of Bryson (1982, 1983). All calves ranged between 3-1/2 and 4-1/2 weeks of age at the time of experimental infection. Calves (Groups II, III, IV) were inoculated twice daily over a period of 4 days. In the morning, calves received 10 ml of virus intranasally (5 ml in each nostril), and 10 ml of virus intratracheally. In the afternoon, virus was administered only by the intranasal route. Calves were made to hyperventilate by forced rebreathing into a small plastic bag prior to inoculation. Control calves (Group I) were also inoculated by the combined respiratory tract route. Non-infected flasks of bovine turbinate cells were processed similarly as described and utilized as inoculum for the control animals.

6. Post-Inoculation Monitoring and Sample Collection

Following experimental infection, calves were observed twice daily (AM and PM) for the development of clinical signs and respiratory tract disease. Body temperature, pulse, and respiratory rates were evaluated at similar times each day and recorded.

A daily arterial blood sample was obtained from the surgically isolated carotid artery on each calf for determination of arterial blood gas

tensions. The sample was collected with a 1 cc tuberculin syringe and a 25 gauge needle (Becton Dickinson & Company, Rutherford, NJ). Sodium heparin (1,000 USP units/ml) (LyphoMed, Inc., Melrose Park, IL) was used in the syringe as an anti-coagulant. Following collection, syringes were capped with a stainless steel female luer (The Micro Group, Franklin, MA). All samples were placed on ice and analyzed within sixty minutes. A blood gas analyzer (ABL1 acid-base Laboratory, Radiometer A/S, Copenhagen, Denmark) was used to evaluate arterial blood gas tensions. Blood gas tensions were measured at 37°C, and corrected to body temperature by the method of Severinghaus (1965).

A venous blood sample, collected with an EDTA-Vacutainer tube (Becton Dickinson & Company, Rutherford, NJ), was used to determine PCV, total protein, fibrinogen, and total leukocyte count with differential. PCV was determined by the microhematocrit method. Total plasma protein was determined by refractometry, while plasma fibrinogen concentration was estimated by heat precipitation and refractometry as described by Jain (1986). Total leukocyte count was performed with an electronic Coulter counter (Model S-Senior, Coulter Electronics, Hialeah, FL), and differential count was determined by microscopic examination of a Wright-stained blood film.

Blood samples for viral serology were collected from each calf prior to infection, and then weekly thereafter until the time of post-mortem examination. Samples were collected in 10 ml silicone-coated Vacutainer tubes containing no additives (Becton Dickinson & Company, Rutherford, NJ). Samples were centrifuged after blood had clotted within the tube. Serum was poured into individual sterile plastic tubes (Falcon 2054, Becton Dickinson Labware, Oxnard, CA), and heat inactivated at

56°C for 30 minutes. Samples were frozen and stored at -20°C. All serotesting was undertaken at the conclusion of the experiment.

Nasal swabs (Dacron Swab, American Scientific Products, McGraw Park, IL) were collected from Group I (control) and Group III calves for virus isolation procedures. Samples were collected 24 hours prior to inoculation, 24 hours post-inoculation (PI), and every third day up to 16 days PI. After wiping the external nares clean with gauze sponges, swabs were inserted full length into the nasal cavity. Swabs were gently rotated for approximately five seconds, removed, and then placed in 2 ml of a transport media consisting of minimal essential medium supplemented with 10% horse serum, gentamicin sulfate (25 ug/ml), sodium penicillin G (50 ug/ml), streptomycin sulfate (50 ug/ml), neomycin sulfate (100 ug/ml), and amphotericin B (2.5 ug/ml). Samples were immediately transported to the laboratory for processing.

Nasal swabs were also obtained from Group I (control) and Group III calves to evaluate detection of BRSV antigen with an Enzyme-Linked Immunosorbent Assay (ELISA)(ORTHO RSV Antigen ELISA Test, Ortho Diagnostic Systems, Raritan, NJ) originally designed for human specimens. Specimens for ELISA testing were collected according to the schedule and technique previously described for virus isolation procedures. ELISA samples were placed in phosphate buffered saline and stored at -70°C until testing was performed.

7. Serologic Studies

Serology for BRSV was performed by a microtiter serum neutralization test. The tests were carried out in Nunculon 96-well microtiter plates (Inter Med, Roskilde, Denmark). Serum (0.5 ml) was dispensed into the first horizontal row of test wells. Growth media with 10% horse

serum (.025 ml) was added to the remaining wells. Serial two-fold dilutions of the serum (from 1:2 to 1:128) were made down the plate with a Multi-Microdiluter Handle fitted with .025 ml Microdiluter capillary transfer pipettes (Dynatech Laboratories, Inc., Alexandria, VA). Five hundred $TCID_{50}$ of test virus (375L isolate of BRSV) was added to all wells except the first horizontal row and an antibody-antigen interaction was allowed to occur at room temperature for one hour. Bovine turbinate cells (.05 ml) were then allocated to all wells at a concentration calculated to form a monolayer in 24 hours. Microtiter plates were covered with sealing tape (Dynatech Laboratories, Inc., Alexandria, VA), and were incubated for five days at 37°C with 5% CO_2. The serum-virus neutralizing titer was determined by the highest dilution at which none or only minimal cytopathic effect was present. Positive and negative control sera were run simultaneously with all test samples.

8. Postmortem Examination and Sample Collection

Postmortem examination was conducted on all calves following euthanasia by barbiturate overdose (Fatal-Plus, Vortech Pharmaceuticals, Dearborn, MI). Immediately after death, the respiratory tract was removed from the thorax and weighed, and gross lesions were recorded. Photographic records were made of the dorsal and ventral surfaces of all pulmonary specimens.

Sections of bronchial lymph node, right cranial lung lobe and left caudal lung lobe were collected for bacterial culture, Mycoplasma and Ureaplasma culture, virus isolation, and direct immunofluorescent examination. The caudal portion of the left cranial lung lobe, bronchial lymph node, tracheal ring, and nasal turbinate were collected for histologic examination.

9. Bacterial Culture

The surface of each tissue specimen was seared with a hot spatula and incised with a sterile scalpel. A sterile, cotton-tipped applicator (American Scientific Products, McGaw Park, IL) was inserted into the surface incision. The sterile swab was then used to inoculate solid culture media (blood agar and MacConkey agar) in petri dishes. The inoculated plates were incubated at 37°C with 5% CO_2. Resultant bacterial colonies were identified biochemically following standard procedures by the Animal Health Diagnostic Laboratory at Michigan State University.

10. Mycoplasma Culture

Mycoplasma culture and identification were performed by the Animal Health Diagnostic Laboratory at Michigan State University. Briefly, tissue specimens were sampled with sterile swabs as previously described. For isolation, swabs were inoculated onto Hayflicks Mycoplasma media and onto Davies Mycoplasma media. In addition, minced tissue samples were inoculated into tubes of Hayflicks Mycoplasma broth and Davies Mycoplasma broth. Plates and tubes were incubated at 37°C with 8% CO_2, and were examined for growth every 48-72 hours. Growth in broth was confirmed by plating. Positive identification of Mycoplasma colonies was confirmed by indirect immunofluorescence. Inoculated plates and tubes were considered negative if no growth occurred within 15 days.

11. Ureaplasma Culture

Ureaplasma culture and identification was performed by the Animal Health Diagnostic Laboratory at Michigan State University. Briefly, tissue specimens were sampled with sterile swabs as previously described. For isolation, swabs were inoculated onto Ureaplasma agar and plates were

anaerobically incubated at 37°C in a GasPak jar containing a GasPak anaerobic system (BBL Microbiology Systems, Cockeysville, MD). Plates were examined every 48 hours for growth. In addition, minced tissue samples were inoculated into tubes of Ureaplasma broth. Tubes were incubated at 37°C with 8% CO_2. Growth in broth was confirmed by plating. Inoculated plates and tubes were considered negative if no growth occurred within 8 days.

12. Virus Isolation

For nasal swab specimens, 1 ml of the transport media was inoculated onto bovine turbinate cells within 1 hour of collection. Bovine turbinate cells were grown on coverslips in Leighton tubes (Wheaton Scientific, Millville, NJ). Viral adsorption was allowed to occur for 1 hour at room temperature. The cell layer was then washed three times with Hanks balanced salt solution (HBSS) and minimal essential medium containing 10% horse serum and standard antibiotics was added. The cell layer was observed daily for virus-induced cytopathic effect. Direct immunofluorescence was used to confirm the identity of isolated viruses.

Routine virus isolation on tissue specimens was performed by the Animal Health Diagnostic Laboratory at Michigan State University. Tissue samples were ground with a sterile mortar, pestle, and sand in 4 ml of minimal essential medium. The homogenate was centrifuged (600 x g for 20 minutes) and then filtered through a Millex-HA filter unit (.45 um pore size) (Millipore Corporation, Bedford, MA). The filtrate was used to inoculate 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

5 days, coverslips were stained with a direct fluorescent antibody conjugate against BVDV (National Animal Disease Laboratory, Ames, IA) to screen for the presence of noncytopathic BVDV. If no cytopathic effect was observed at the end of 7 days, the tissue sample was reported as negative.

13. ELISA

The BRSV isolate used to infect calves was determined to react in the ELISA test prior to infection studies. Nasal swab specimens from control (Group I) and principal (Group III) calves were collected and assayed according to manufacturer's protocol (ORTHO RSV Antigen ELISA Test, Ortho Diagnostic Systems, Raritan, NJ).

14. Fluorescent Antibody Examination

Tissue specimens were placed into 5 ml polystyrene beakers (diSPo Beaker, American Scientific Products, McGaw Park, IL) containing O.C.T. embedding compound (Miles Laboratories, Inc., Naperville, IL) and stored at -70^{o}C until further processing. Eight micron-thick tissue sections were cut on a cryostat microtome (Minotome, International Equipment Co., Needham Heights, MA) and then mounted onto glass microscope slides. The slides were fixed in acetone for 10 minutes, rinsed in distilled water, washed in phosphate buffered saline (pH 7.2) for 5 minutes, rinsed again, and air-dried. The tissue sections were encircled with Marktex ink (Mark-Tex Corp., Englewood, NJ) and fluorescent antibody conjugates specific for infectious bovine rhinotracheitis virus, BVDV, parainfluenza-3 virus (National Animal Disese Laboratory, Ames, IA) and BRSV (Dr. Merwin L. Frey, University of Nebraska) were applied. Slides were incubated in a humid chamber at 37^{o}C for 30 minutes. Following incubation, the

conjugate was decanted and the slides were washed in phosphate buffered saline (pH 8.2) for 5 minutes, rinsed in distilled water, counterstained in 0.01% Evan's blue (Sigma Diagnostics, St. Louis, MO), rinsed again, and then air-dried. A coverslip was mounted onto the slide with 90% glycerol in phosphate buffered saline, and the specimens were examined microscopically using incident UV illumination (Carl Zeiss, Oberkochen, West Germany).

15. Histologic Techniques

The major bronchus leading to the caudal portion of the left cranial lung lobe was cannulated with polyethylene tubing and the lobe fixed in 10% formalin by a combination of submersion and airway perfusion at 30 cm of fixative pressure. Bronchial lymph node, tracheal ring, and nasal turbinate were also fixed by submersion in formalin. All fixed tissues were embedded in paraffin, sectioned at 5 microns, stained with hematoxylin and eosin, and examined by light microscopy.

16. Pulmonary Surface Area Measurements

At necropsy, 35 millimeter color slides (Ektachrome, Kodak Co., Rochester, NY) were made of the dorsal and ventral surfaces of all gross pulmonary specimens. A metric scale was included in each photograph. The slides were projected onto white copier paper, and an exact tracing of the pulmonary image, including the metric reference scale, was recorded. Areas of normal and pneumonic lung tissue were quantified from the tracing using a high resolution (0.025 mm [0.001 inch]) opaque digitizing board (Jandel Scientific, Sausalito, CA) connected to an IBM XT computer with appropriate software (Sigma-Scan, Jandel Scientific, Sausalito, CA). Results were reported in square centimeters.

Calibrated areas were determined for each pneumonic region, summed, and subsequently referenced to total lung area.

17. Statistical Analysis

Treatment means were statistically analyzed in an effort to detect the presence of real differences among the four experimental groups. Statistical evaluation was performed on the following experimental data: body temperature, heart rate, respiratory rate, total protein, plasma fibrinogen, white blood cell count, segmented neutrophils, lymphocytes, monocytes, arterial oxygen tension (P_aO_2), arterial carbon dioxide tension (P_aCO_2) and pneumonic involvement of total pulmonary surface area (%).

Some data sets required transformation to correct for non-normality (Steel and Torrie, 1980). Logarithmic transformation was performed on white blood cell, neutrophil, lymphocyte and monocyte counts. An arcsin transformation was utilized on pneumonic surface area data.

All analyses of variance were computer generated according to the General Linear Model procedure described by the SAS Institute (SAS Institute, Inc., 1985). Body temperature, heart rate, respiratory rate, total protein, plasma fibrinogen, white blood cell count, segmented neutrophils, lymphocytes, monocytes, P_aO_2, and P_aCO_2 were evaluated by a factorial split plot analysis of variance corrected for repeat measures (Steel and Torrie, 1980). Two different analyses were required in order to correctly examine all possible comparisons. Experimental groups I (control), II, III and IV were compared by evaluating experimental data collected between days 1 through 8. Experimental groups I (control), III and IV were compared by evaluating experimental data collected between days 1 and 24. When significant differences ($p < 0.05$) between means

were discovered, individual treatment - day means were compared by Tukey's procedure.

Pneumonic involvement of total pulmonary surface area was statistically analyzed by a one-way analysis of variance (Steel and Torrie, 1980). Significant differences between experimental groups were also compared by Tukey's procedure.

RESULTS

1. Clinical Signs

Respiratory tract disease was produced in groups II, III, and IV. Clinical signs initially appeared 1-2 days after the final day of viral inoculation, peaked by 4-5 days post-inoculation, and generally resolved by 6-8 days post-inoculation. Intensity of clinical signs varied considerably among calves within a group. In some calves, signs of respiratory tract disease were mild and transient, whereas in others severe signs of respiratory distress predominated. One calf (124) in Group IV developed fatal bronchopneumonia during this trial. This calf was excluded from data analysis and statistical consideration.

The clinical signs observed in experimentally infected calves during this study included pyrexia, tachypnea, hyperpnea, dyspnea, depression, lethargy, excessive lacrimation, nasal and ocular discharges, hypersalivation, and coughing. Anorexia usually was not part of the induced clinical syndrome. Most calves did not refuse to nurse even during periods of respiratory distress.

Clinical signs of respiratory tract disease were never observed in Group I (control) calves during this study. In Group II, all calves displayed clinical signs following viral inoculation. Severe dyspnea and depression were produced in four of the six calves comprising this group. In Group III, all six calves also developed signs of respiratory tract infection. In four calves, signs were mild and transient. However, in the remaining two calves, signs of severe respiratory tract

disease persisted for approximately 72 to 96 hours following inoculation. Extreme dyspnea interfered with the ability of these two calves to nurse. In Group III calves, respiratory tract disease was monophasic, and calves appeared to be recovered by 7-8 days post-inoculation. In Group IV calves, initial inoculation produced mild to moderate signs of respiratory tract disease. The clinical signs resolved during the interim period between inoculations, and rechallenge failed to induce or exacerbate signs of respiratory tract disease. One Group IV calf (124) developed fatal bronchopneumonia following the initial viral inoculation. Unlike the remainder of the group, this calf displayed severe signs of respiratory tract disease.

2. Clinical and Clinicopathological Response

A. Body Temperature (^{o}C)

1) Groups I, II, III, and IV (Days 1-8)

Following inoculation, mean body temperature increased with time in all groups (Figure 1A). Temperature elevations in the control group, however, never exceeded the accepted physiological range for normal calves (37.8-39.2^{o}C)(Rosenberger, 1979). Mean body temperatures were consistently found to be greater in all treatment groups when compared to the control group throughout the entire experimental period. The greatest febrile response was observed in Group II on experimental day 7 (mean = 39.96^{o}C).

The differences in body temperature between groups were found to be highly significant ($F = 5.86$, $P < 0.0052$). Significant differences between control and treatment groups are indicated in Figure 1A. Significant differences were observed between control and treatment groups

II, III and IV on days 4 through 8. Significant differences were most numerous between the control and Group II.

2) Groups I, III and IV (days 1-24)

Following inoculation, mean body temperature increased with time in all groups (Figure 1B). Body temperature elevations, however, were substantially greater in treatment groups relative to the control group. Mean temperature elevations in the control group never exceeded the accepted physiological range for normal calves (37.8-39.2^{o}C)(Rosenberger, 1979). The greatest febrile response was observed in Group III calves on experimental day 7 (mean = 39.73^{o}C).

In Group III, mean body temperature remained elevated for approximately one week following BRSV inoculation. Thereafter, the daily mean body temperature of Group III approached and closely followed control values for the remainder of the experimental period. In Group IV, rechallenge exposure to BRSV appeared to sustain the initial observed elevation in mean body temperature. Group IV values, however, also approached control levels by the conclusion of the experiment.

The observed differences in body temperature between groups proved to be significant ($F = 5.27$, $P < 0.0197$). Significant differences between control and treatment groups are indicated in Figure 1B. Following initial viral inoculation, significant differences in mean body temperature existed between control and treatment groups on experimental days 4 through 10. BRSV rechallenge exposure in Group IV calves appeared to prolong this period of significance. BRSV rechallenge also produced significant differences in body temperature between Group III and Group IV calves on experimental days 17, 19, and 20.

Figure 1A: Effect of BRSV infection on body temperature (^{o}C) in groups I, II, III, and IV, days 1-8. Arrows indicate administration of inoculum; all groups were inoculated on days 1-4. Asterisks indicate significant differences ($P < 0.05$) between control and principal groups by Tukey's test. Tukey's critical values for between group comparisons are demonstrated. Due to unequal replication in Group IV, critical value (b) should be used in all comparisons involving Group IV calves. Use critical value (a) for all other between group comparisons.

Figure 1B: Effect of BRSV infection on body temperature (^{o}C) in groups I, III, and IV, days 1-24. Arrows indicate administration of inoculum; all groups were inoculated on days 1-4; Group IV calves were rechallenged with BRSV on days 14-17. Asterisks indicate significant difference ($P < 0.05$) between control and principal groups by Tukey's test. A plus sign indicates a significant difference ($P < 0.05$) between Group III and Group IV by Tukey's test. Tukey's critical values for between group comparisons are demonstrated. Due to unequal replication in Group IV, critical value (b) should be used in all comparisons involving Group IV calves. Use critical value (a) for all other between group comparisons.

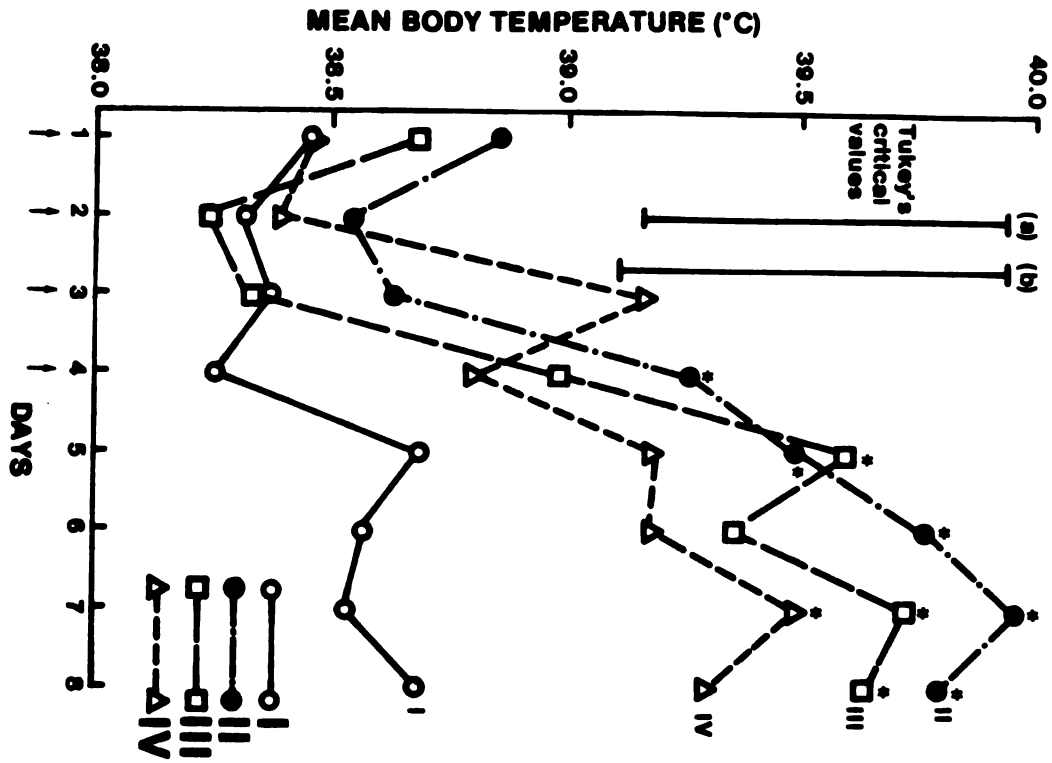

Figure 1A

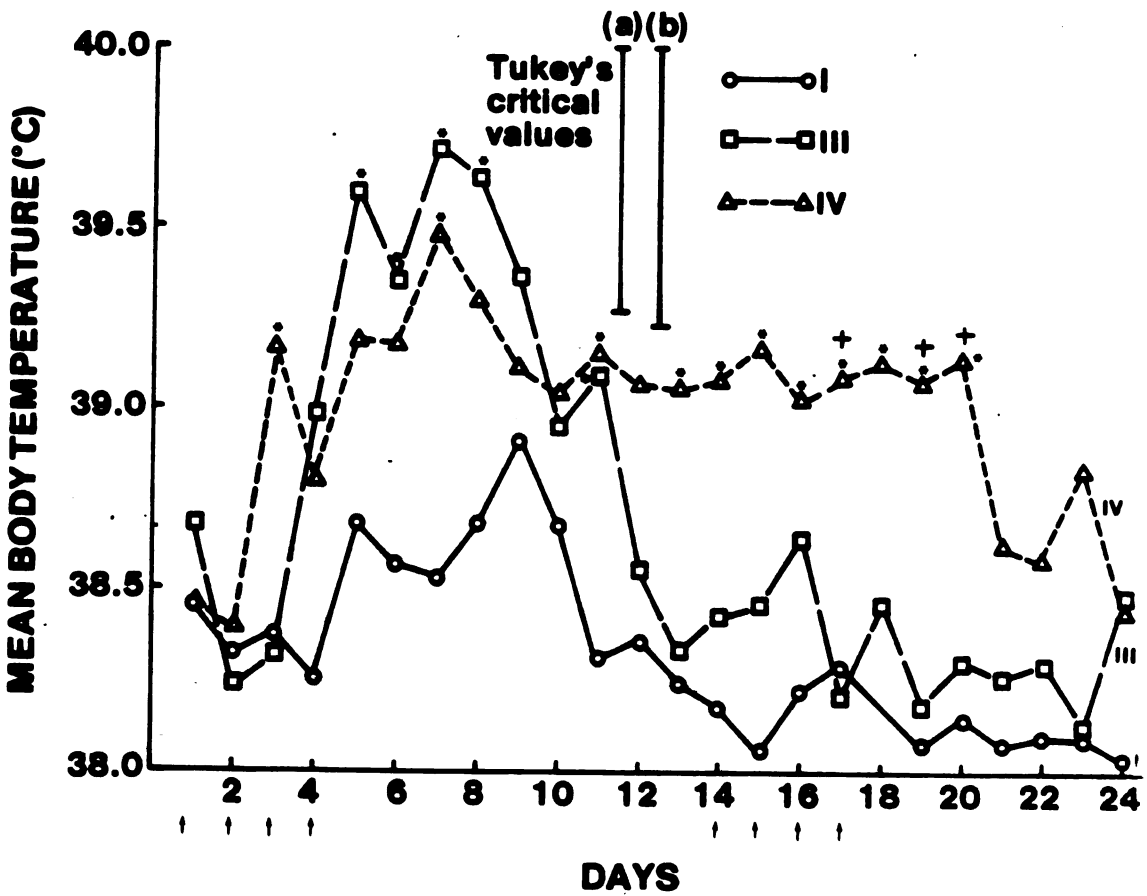

Figure 1B

B. Heart Rate (beats per minute).

1) Groups I, II, III and IV (Days 1-8)

Following inoculation, mean heart rate increased with time in all groups (Figure 2A). Heart rate elevations in the control group, however, never exceeded the accepted physiological range for young calves (70-90 beats per minute)(Rosenberger, 1979). Mean heart rate was consistently found to be greater in all treatment groups relative to the control throughout the entire experimental period. Calves in groups II and III demonstrated larger increases in heart rate than calves in Group IV. The largest increase in mean heart rate was observed in Group II calves on experimental day 6 (mean = 116.0 $\pm$ 9.8 beats per minute).

The differences in mean heart rate between groups were found to be highly significant ($F = 5.57$, $P < 0.0065$). Significant differences between control and treatment groups are indicated in Figure 2A. Significant differences in mean heart rate were observed between control and treatment groups II and III on experimental days 5 through 8. No significant differences in mean heart rate existed between control and treatment Group IV.

2) Groups I, III, and IV (Days 1-24)

Although differences in mean heart rate existed between groups I, III, and IV, none of these differences proved to be statistically significant ($F = 2.41$). Mean heart rate increased in all experimental groups following inoculation (Figure 2B). Elevations observed in control animals never exceeded the accepted physiological range for young calves (70-90 beats per minute)(Rosenberger, 1979). The largest elevation in mean heart rate was observed in Group III calves on experimental day 8 (mean = 115.33 $\pm$ 21.68 beats per minute). By day 13, mean heart rates

Figure 2A: Effect of BRSV infection on mean heart rate (beats per minute) in groups I, II, III, and IV, days 1-8. Arrows indicate administration of inoculum; all groups were inoculated on days 1-4. Asterisks indicate significant differences ($P < 0.05$) between control and principal groups by Tukey's test. Tukey's critical values for between group comparisons are demonstrated. Due to unequal replication in Group IV, critical value (b) should be used in all comparisons involving Group IV calves. Use critical value (a) for all other between group comparisons.

Figure 2B: Effect of BRSV infection on mean heart rate (beats per minute) in groups I, III, and IV, days 1-24. Arrows indicate administration of inoculum; all groups were inoculated on days 1-4; Group IV calves were rechallenged with BRSV on days 14-17. Although differences in heart rate existed between control and principal groups, none of the observed differences proved to be statistically significant.

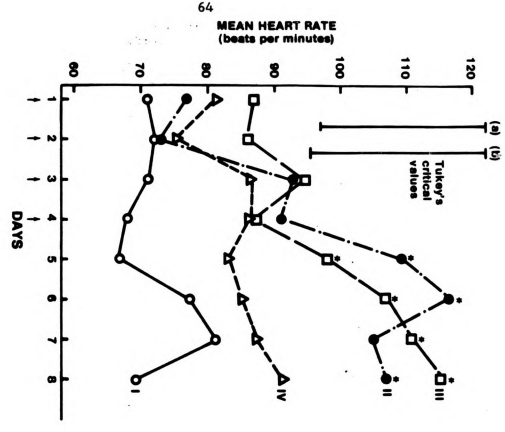

Figure 2A

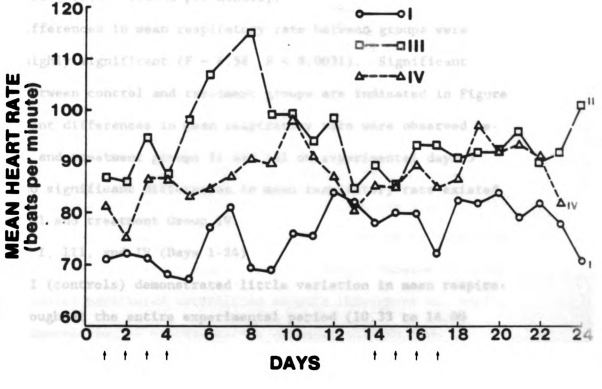

Figure 2B

in both treatment groups decreased and approached control levels. However, heart rates remained consistently higher in treatment groups relative to the control throughout the remainder of the experimental period. Rechallenge exposure did not appear to influence mean heart rate in Group IV calves.

C. Respiratory Rate (breaths per minute)

1) Groups I, II, III, and IV (Days 1-8)

Following inoculation, mean respiratory rates increased only in the principal groups (Figure 3A). Sham inoculation did not appear to influence respiratory rate in control calves. Mean respiratory rates of control calves remained within the accepted physiological range for young calves (15-40 breaths per minute)(Rosenberger, 1979). Mean respiratory rates rose sharply in treatment groups II and III. A more modest increase in respiratory rate was observed in Group IV. The largest elevation in mean respiratory rate occurred in Group III on experimental day 8 (mean = 58.33 $\pm$ 36.49 breaths per minute).

The differences in mean respiratory rate between groups were found to be highly significant ($F = 6.58$, $P < 0.0031$). Significant differences between control and treatment groups are indicated in Figure 3A. Significant differences in mean respiratory rate were observed between control and treatment groups II and III on experimental days 5 through 8. No significant differences in mean respiratory rate existed between control and treatment Group IV.

2) Groups I, III, and IV (Days 1-24)

Group I (controls) demonstrated little variation in mean respiratory rate throughout the entire experimental period (10.33 to 16.00

breaths per minute)(Figure 3B). In Group III, mean respiratory rate increased sharply and remained elevated for approximately one week after BRSV infection. Thereafter, mean respiratory rate approached and closely followed control values for the remainder of the experimental period. Group IV demonstrated a moderate increase in respiratory rate following initial viral exposure. Rechallenge exposure appeared to sustain the observed elevation in mean respiratory rate for an additional 5 to 7 days. Respiratory rate in Group IV calves, however, returned to control levels by conclusion of the experiment.

The observed differences in respiratory rate between groups proved to be significant ($F = 5.27$, $P < 0.0197$). Significant differences between control and treatment groups are indicated in Figure 3B. Significant differences in mean respiratory rate were found between control and treatment Group III on experimental days 6 through 10. Periods of significance existed between control and treatment Group IV on experimental days 5 through 8, experimental day 14, and experimental days 18 and 19. Rechallenge exposure of Group IV also produced significant differences in respiratory rate between Group III and Group IV calves on experimental days 14 and 18.

D. Arterial Oxygen Tension (P_aO_2)(mm of Hg)

1) Groups I, II, III, and IV (Days 1-8)

Arterial oxygen tensions in the normal resting calf have been established in previous studies: 81.0 ± 2 mm Hg (Bisgard and Vogel, 1971), 85.7 ± 1.9 mm Hg (Bisgard et al., 1973), and 93.6 ± 7.68 mm Hg (Donawick and Baue, 1968). In the present study, arterial oxygen tension in Group I calves (controls) paralleled established normals throughout the entire study. Sham inoculation of control calves appeared only to have a

Figure 3A: Effect of BRSV infection on mean respiratory rate (breaths per minute) in groups I, II, III, and IV, days 1-8. Arrows indicate administration of inoculum; all groups were inoculated on days 1-4. Asterisks indicate significant differences ($P < 0.05$) between control and principal groups by Tukey's test. Tukey's critical values for between group comparisons are demonstrated. Due to unequal replication in Group IV, critical value (b) should be used in all comparisons involving Group IV calves. Use critical value (a) for all other between group comparisons.

Figure 3B: Effect of BRSV infection on mean respiratory rate (breaths per minute) in groups I, III, and IV, days 1-24. Arrows indicate administration of inoculum; all groups were inoculated on days 1-4; Group IV calves were rechallenged with BRSV on days 14-17. Asterisks indicate significant differences ($P < 0.05$) between control and principal groups by Tukey's test. A plus sign indicates a significant difference ($P < 0.05$) between Group III and Group IV calves by Tukey's test. Tukey's critical values for between group comparisons are demonstrated. Due to unequal replication in Group IV, critical value (b) should be used in all comparisons involving Group IV calves. Use critical value (a) for all other between group comparisons.

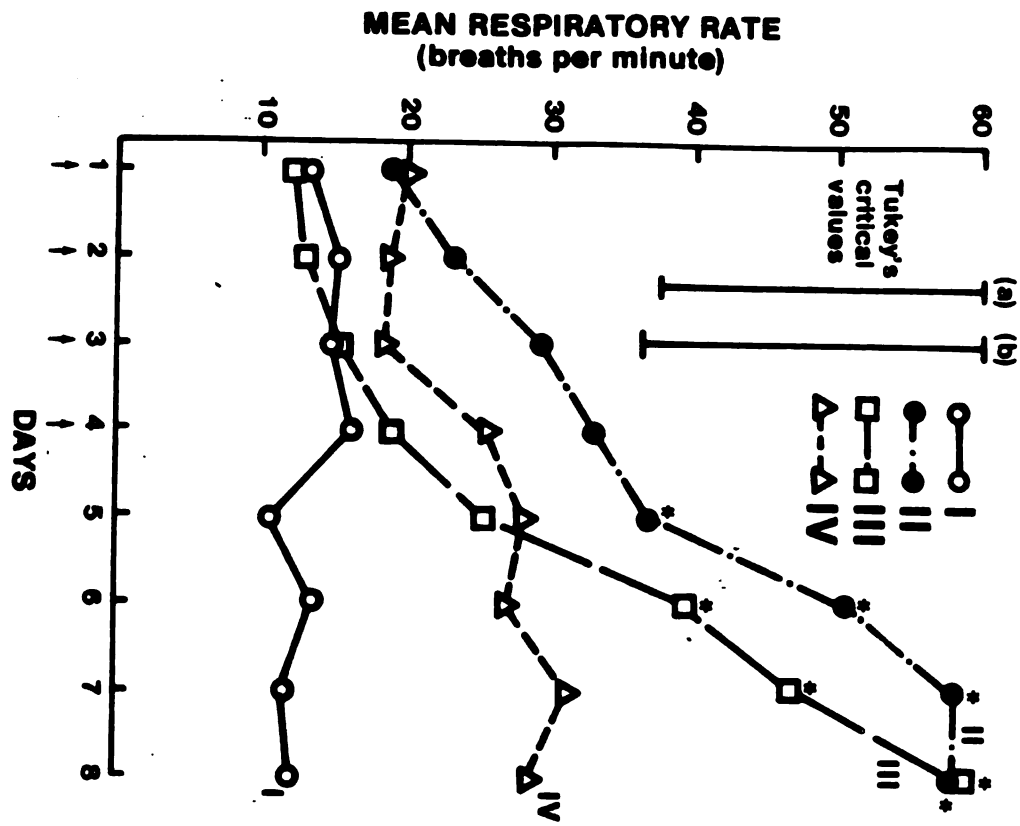

Figure 3A

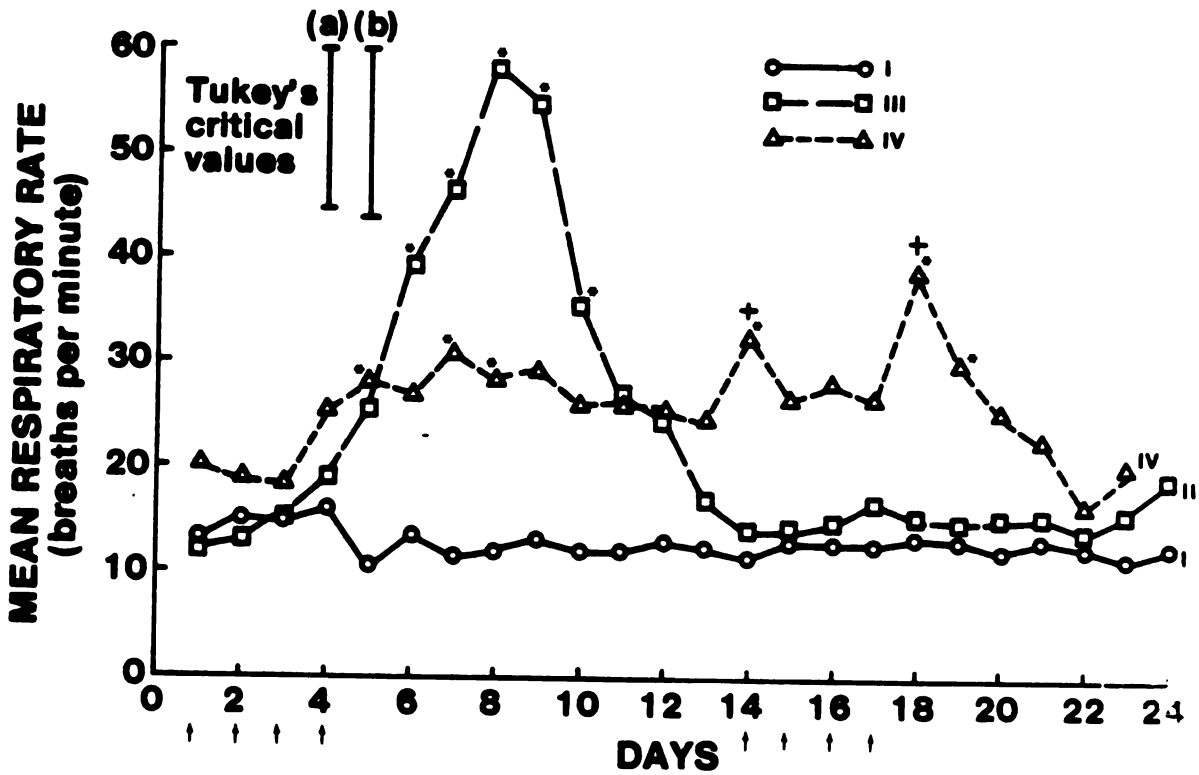

Figure 3B

minimal effect on P_aO_2. Arterial oxygen tension, however, decreased substantially in all treatment groups (groups II, III, IV) subjected to BRSV inoculation (Figure 4A). Mean P_aO_2 levels were consistently lower in all experimentally-infected groups relative to control animals and the lowest mean P_aO_2 was observed in Group III on experimental day 6 (66.97 $\pm$ 4.43 mm Hg).

The differences in P_aO_2 between groups were found to be highly significant ($F = 6.10$, $P < 0.0057$). Significant differences between control and treatment groups are indicated in Figure 4A. Significant differences in mean P_aO_2 were observed between control and treatment groups II, III and IV on experimental days 6 through 8. Significant differences were most numerous between the control group and treatment group III.

2) Groups I, III, and IV (Days 1-24)

Although differences in mean arterial oxygen tension existed among experimental groups, none of these differences proved to be statistically significant ($F = 2.75$)(Figure 4B). P_aO_2 values in Group I calves (controls) corresponded with established normals throughout the entire experimental period. P_aO_2 decreased in each of the experimentally-infected groups for approximately 8 days following viral inoculation. Thereafter, P_aO_2 levels in Group III and Group IV approached and closely matched control values for the remainder of the experimental period. Rechallenge exposure of Group IV failed to influence P_aO_2 levels.

E. Additional Clinicopathological Findings

White blood cell count, differential leukocyte count, packed cell volume, plasma protein concentration, plasma fibrinogen concentration,

Figure 4A: Effect of BRSV infection on mean arterial oxygen tension (corrected for body temperature) in groups I, II, III, and IV, days 1-8. Arrows indicate administration of inoculum; all groups were inoculated on days 1-4. Asterisks indicate significant difference ($P < 0.05$) between control and principal groups by Tukey's test. Tukey's critical values for between group comparisons are demonstrated. Due to unequal replication in Group IV, critical value (b) should be used in all comparisons involving Group IV calves. Use critical value (a) for all other between group comparisons.

Figure 4B: Effect of BRSV infection on mean arterial oxygen tension (corrected for body temperature) in groups I, III, and IV, days 1-24. Arrows indicate administration of inoculum; all groups were inoculated on days 1-4; Group IV calves were rechallenged with BRSV on days 14-17. Although differences in P_aO_2 existed between control and treatment groups, none of the observed differences proved to be statistically significant.

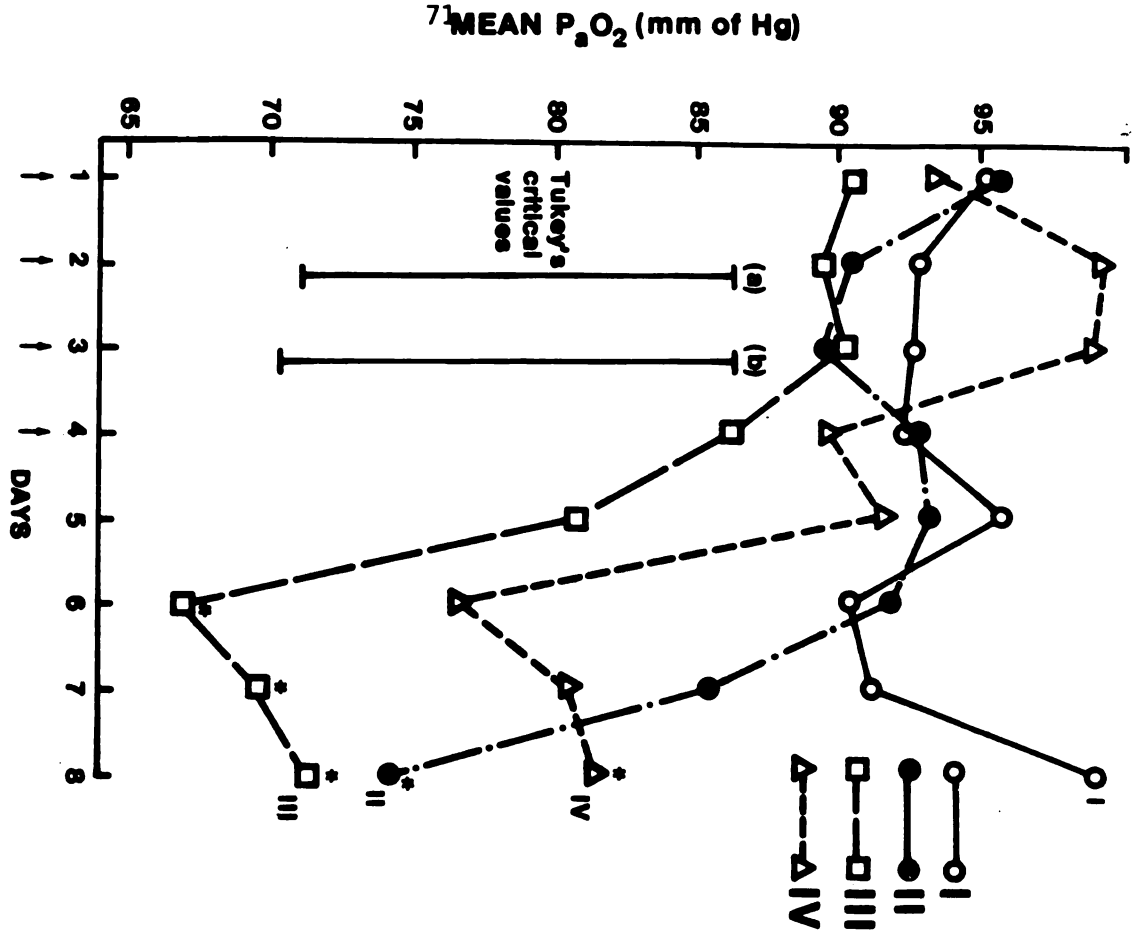

Figure 4A

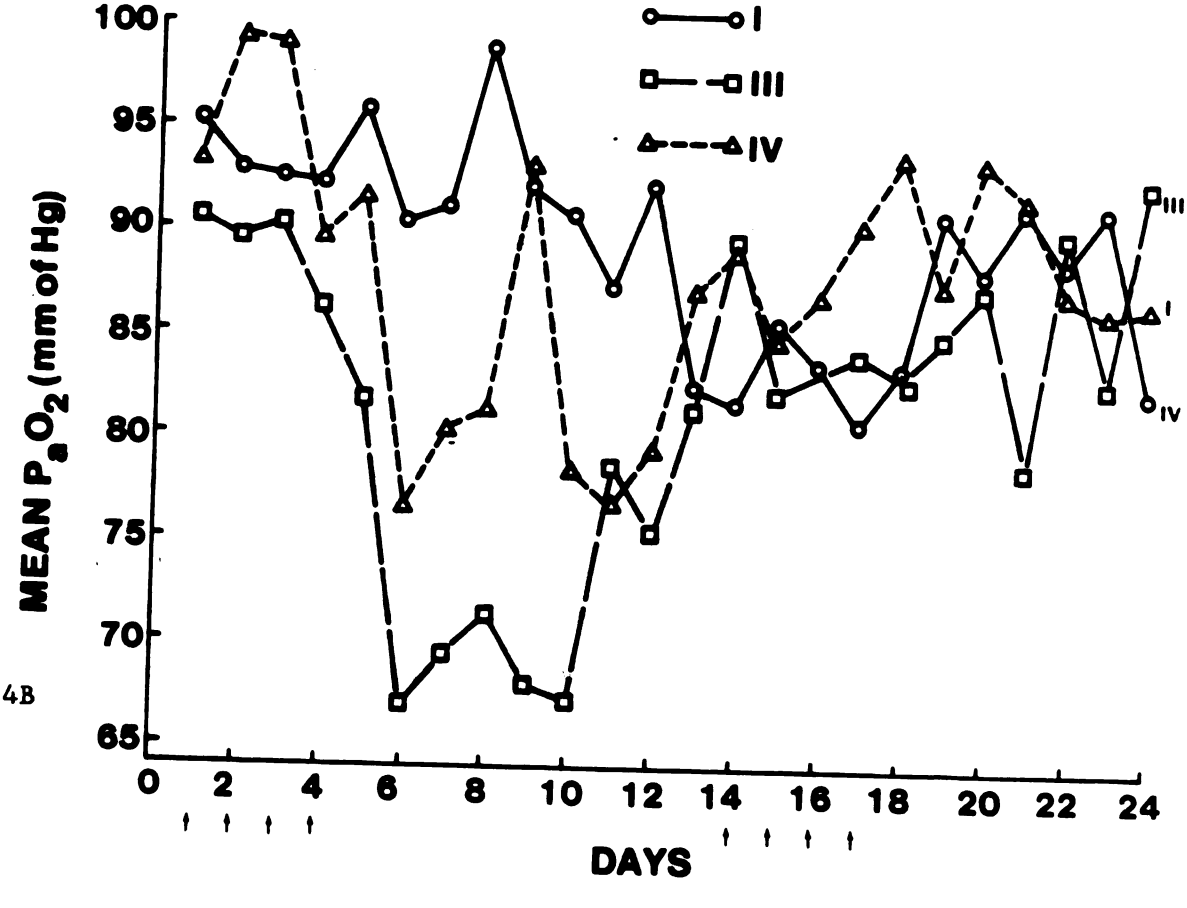

Figure 4B

and arterial carbon dioxide tension (P_aCO_2) were determined on a daily basis for all experimental subjects. Experimental infection with BRSV did not markedly influence these variables. There was no deviations or trends observed between the principal groups as compared to the control group nor was there significant differences detected in any of these variables between the four groups.

3. Serologic Studies

Serum neutralizing antibody titers to BRSV for the four groups of calves are listed in Tables 1 through 4. Each calf, with one exception (124), was found to possess serum antibody against BRSV. Antibodies were most likely passively derived and individual calf titers ranged from 1:2 to 1:64. Serum antibody titers remained relatively constant in all calves throughout the experimental period (Figure 5) and no calf, aside from 124, displayed seroconversion to BRSV (a minimal increase in serum antibody titer of at least four-fold) following experimental infection. The only calf (124) lacking antibody to BRSV at the time of inoculation was also the only calf to develop fatal bronchopneumonia during this research trial.

4. BRSV Isolation and ELISA-Nasal Swabs

Virus isolation and ELISA techniques failed to detect BRSV in nasal swab samples collected from Group I calves (controls).

In Group III calves, BRSV was not demonstrated from pre-inoculation nasal swabs by either virus isolation or ELISA procedures. However, BRSV was isolated from the majority of Group III calves on days 1, 4 and 7 post-inoculation. The ELISA test only detected BRSV in one Group III calf at day 1 post-inoculation.

Table 1. Reciprocal serum neutralizing antibody titers to BRSV in Group I (control) calves. Calves were inoculated with BRSV on days 1 through 4.

Calf Number	Day 1 (Preinoculation)	Day 8	Day 15	Day 22
102	16	16	8	16
103	4	4	2	4
104	4	4	2	2
105	8	16	16	8
106	4	4	2	2
109	8	8	8	8

Table 2: **Reciprocal serum neutralizing antibody titers to BRSV in Group II calves. Calves were inoculated with BRSV on days 1 through 4.**

Calf Number	Day 1 (Preinoculation)	Day 7
115	32	32
116	64	32
117	4	4
118	32	32
119	32	32
120	32	32

Table 3: Reciprocal serum neutralizing antibody titers to BRSV in Group III calves. Calves were inoculated with BRSV on days 1 through 4.

Calf Number	Day 1 (Preinoculation)	Day 5	Day 12	Day 19	Day 24
107	2	4	2	2	2
108	64	32	32	32	32
110	2	2	2	4	4
111	32	32	32	32	64
113	16	32	4	8	16
114	8	8	4	8	8

Table 4: Reciprocal serum neutralizing antibody titers to BRSV in Group IV calves. Calves were inoculated with BRSV on days 1 through 4 and rechallenged on days 14 through 17.

Calf Number	Day 1 (Preinoculation)	Day 9	Day 16	Day 22
121	32	32	16	16
122	16	16	8	8
123	8	4	4	4
124	neg	4	4	*
125	8	16	8	8
126	2	4	4	4

neg = negative

* = died

Figure 5: **Geometric mean serum antibody titer to BRSV in groups I, II, III, and IV. Arrows indicate administration of inoculum; all groups were inoculated on days 1-4; Group IV calves were rechallenged with BRSV on days 14-17.**

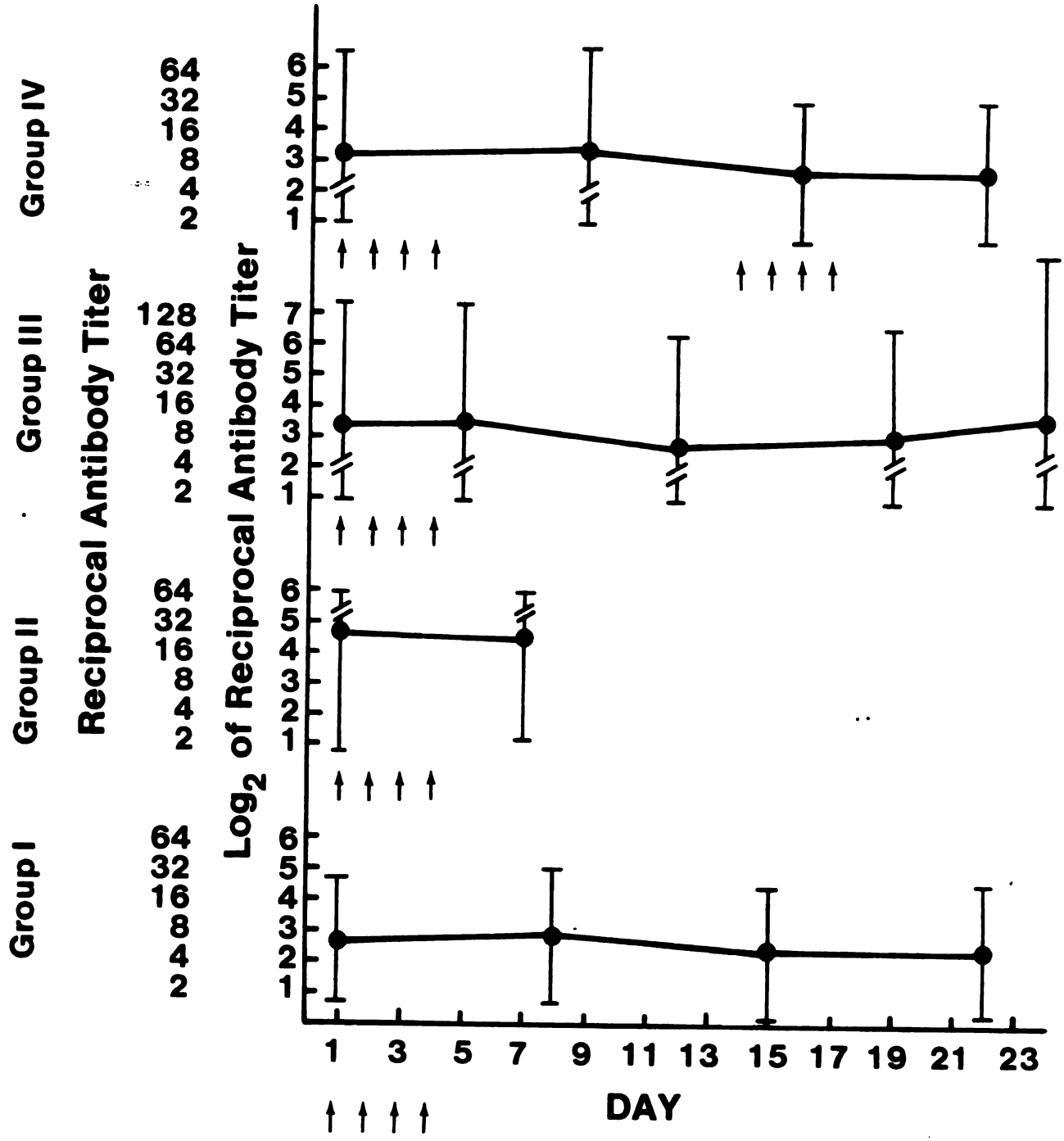
Group IV
Group III
Group II
Group I
Reciprocal Antibody Titer
64
32
16
8
4
2
128
64
32
16
8
4
2
64
32
16
8
4
2
64
32
16
8
4
2
Log2 of Reciprocal Antibody Titer
6
5
4
3
2
1
7
6
5
4
3
2
1
6
5
4
3
2
1
6
5
4
3
2
1
1 3 5 7 9 11 13 15 17 19 21 23
DAY

Figure 5

5. Postmortem Examination

A. Microbiology - Tissue Specimens

1) Bacteriology

Group I - No pulmonary pathogens were cultured from lung and lymph node specimens. Pasteurella multocida was isolated from a pulmonary abscess in calf 106.

Group II - Pasteurella multocida was isolated from the right cranial lung lobe of calf 119 and from the bronchial lymph node of calf 120.

Group III - No pulmonary pathogens were cultured from lung and lymph node specimens. Actinomyces pyogenes and Pasteurella haemolytica were isolated from a pulmonary abscess located in the right middle lobe of calf 111.

Group IV - Pasteurella multocida was isolated from the lung and Actinomyces pyogenes was isolated from the bronchial lymph node of calf 124.

2) Mycoplasma Culture

Group I, II, III - Mycoplasma sp was not cultured from lung and lymph node specimens.

Group IV - Mycoplasma bovirhinis was isolated from the bronchial lymph node of calf 123.

3) Ureaplasma Culture

Group I, III, IV - Ureaplasma sp was not cultured from lung and lymph node specimens.

Group II - Ureaplasma sp was isolated from the right cranial lobe and left caudal lobe of calf 120.

4) Virus Isolation - Tissue Specimens

No viruses were isolated from lung and lymph node specimens submitted from all control and principal calves.

B. Direct Fluorescent Antibody Examination

Immunofluorescence for IBR, BVD, PI_3 or BRSV was not detected in lung and lymph node sections prepared from all control and principal calves.

C. Gross Pathology

Gross lesions, indicative of respiratory tract disease and viral pneumonia, were observed in each principal group. However, a gross examination of the respiratory tracts from Group I calves (controls) revealed only minimal pathologic changes. In three control calves (103, 104, 106), scattered areas of atelectasis and congestion were present in the lungs (Figure 6). One of these calves (106) also developed a small (10 mm), solitary abscess in the right cranial lung lobe. The remaining three control calves (102, 105, 109) displayed no gross changes in their respiratory tracts (Figure 7).

Gross pathology was most severe in Group II. Extensive areas of hemorrhage and lobular consolidation were concentrated in the cranial and middle lobes (Figures 8 and 9). Consolidated lobules were dark red in color and firm in texture. Three (118, 119, 120) of the six calves in this group had at least one lung lobe which was totally consolidated. Severe consolidation occurred most consistently in the caudal part of the left cranial lung lobe. One calf (120) had fibrinous adhesions between the left cranial lung lobe and the pericardial sac. Another calf (118) had over-inflated, emphysematous caudal lung lobes. Bronchial lymph nodes were enlarged in all calves.

The gross lesions observed in Group III were characteristic of a resolving, viral pneumonia. Gross respiratory tract lesions were minimal and consisted primarily of petechiae and focal areas of consolidation (Figure 10). Enlarged bronchial lymph nodes were also occasionally observed in some calves. One calf (113) had no gross lesions in the respiratory tract. Another calf (111), however, had severe pathologic alterations uncharacteristic of the calves comprising this group.

Figure 6: **Lungs from a Group I calf (104): dorsal surface (A) and ventral surface (B). Scattered areas of congestion (indicated by arrows) are present throughout the lung lobes.**

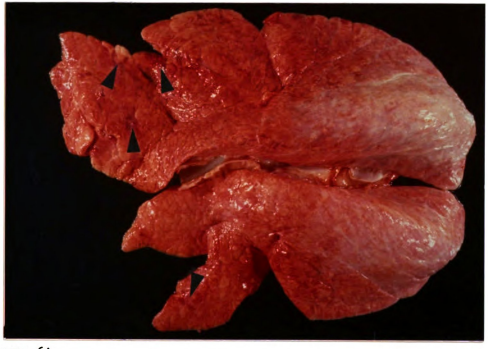

Figure 6A

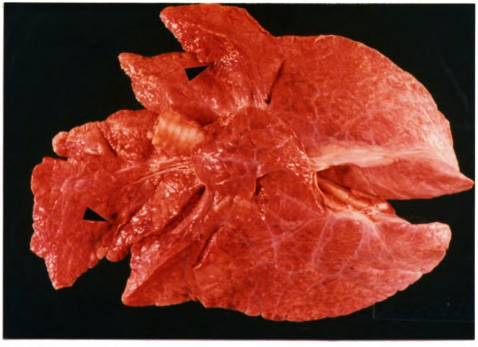

Figure 6B

Figure 7: **Lungs from a Group I calf (102): dorsal surface (A) and ventral surface (B). No gross lesions are present in these views.**

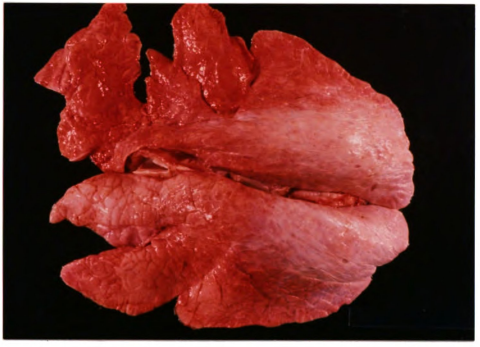

Figure 7A

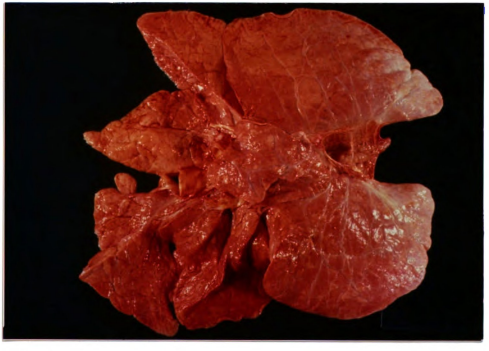

Figure 7B

Figure 8: **Lungs (dorsal surface) from a Group II calf (118). Areas of lobular consolidation are predominantly confined to the cranioventral lung lobes.**

Figure 9: **Lungs (ventral surface) from a Group II calf (118). Widespread areas of lobular consolidation are evident.**

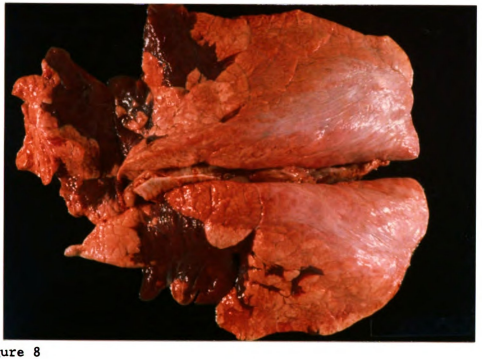

Figure 8

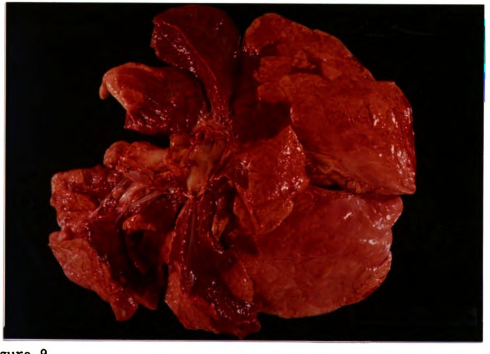

Figure 9

Figure 10: **Lungs from a Group III calf (110): dorsal surface (A) and ventral surface (B). Focal areas of lobular consolidation (indicated by arrows) are present in the left cranial and right middle lung lobes.**

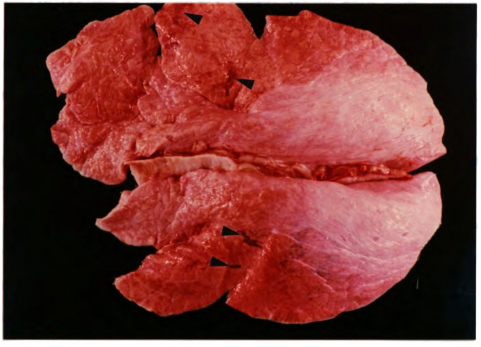

Figure 10A

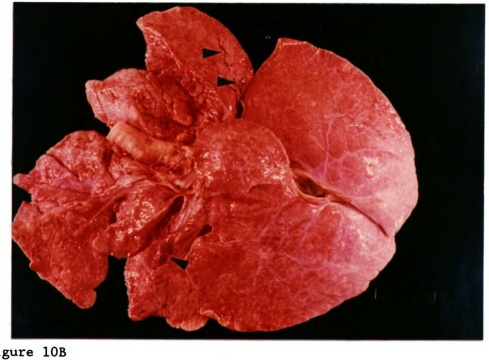

Figure 10B

Extensive regions of pulmonary consolidation were found throughout the lung of this animal (Figure 11). Fibrinous adhesions were present between the cranial and caudal aspects of the right cranial lung lobe. A 25 mm subpleural gas bulla was identified in the right cranial lung lobe. Abscesses were noted in both the right caudal and left cranial lung lobe (Figure 12). The viral pneumonia in this calf was complicated by secondary bacterial invasion.

Gross respiratory tract lesions were relatively mild in Group IV calves. Distinct foci of consolidation, hyperemia and congestion were occasionally observed within the lungs of most calves (Figure 13). Bronchial lymph nodes were enlarged in some cases. In one calf (121), the left caudal lung lobe failed to collapse when the thoracic cavity was opened (Figure 14). This lobe had increased consistency. Multiple cross-sections revealed extensive consolidation in deeper portions of the lung parenchyma that was not apparent on external surface examination. Another calf (124) had severe respiratory tract pathology uncharacteristic of the remainder of the group. This calf developed fatal bronchopneumonia prior to the second viral inoculation and thus was excluded from further study.

D. Pulmonary Surface Area Measurements

Total lung surface area (cm^2) and pneumonic surface area (cm^2) is presented for each calf in Table 5. Pneumonic area was subsequently referenced to total lung area and results are listed in Table 6. Mean pneumonic surface area was determined for each experimental group and group means were statistically compared (Table 6).

Figure 11: Lungs (dorsal surface) from a Group III calf (111). The severe pulmonary consolidation observed in this specimen was uncharacteristic of the respiratory tract lesions generally found in Group III calves.

Figure 12: Lungs (ventral surface) from a Group III calf (111). Pulmonary consolidation is extensive. A 20 mm abscess (small arrow) is present in the right caudal lobe. A 25 mm subpleural gas bulla (large arrow) is found in the caudal part of the right cranial lobe. The caudal lung lobes are hyperinflated.

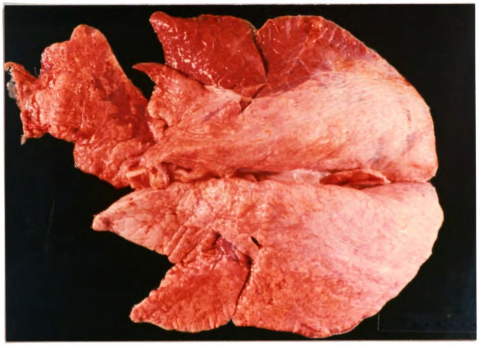

Figure 11

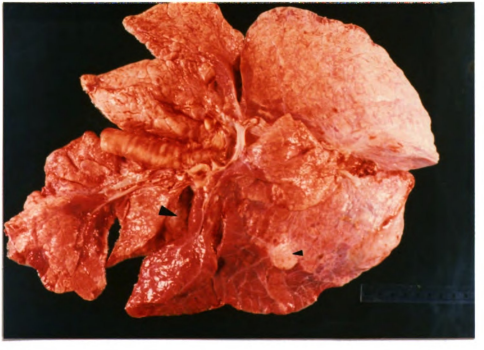

Figure 12

Figure 13: **Lungs from a Group IV calf (122): dorsal surface (A) and ventral surface (B). On dorsal view, consolidated lobules are present in the right cranial, right middle, and left cranial lung lobes. On ventral view, consolidation and congestion are visible in the right cranial lung lobe.**

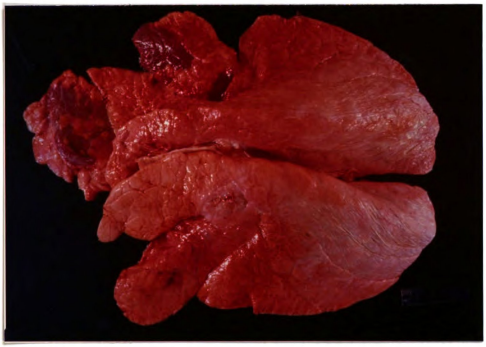

Figure 13A

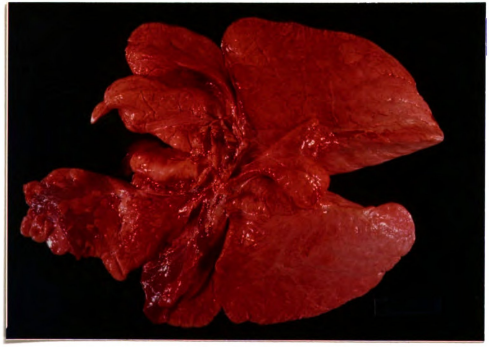

Figure 13B

Figure 14: **Lungs (dorsal surface) from a Group IV calf (121). The left caudal lung lobe failed to collapse when the thoracic cavity was opened and appears hyperinflated. Note the consolidated area in the caudal part of the left cranial lung lobe.**

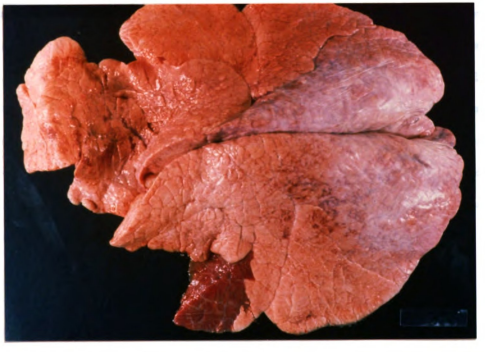

Figure 14

Pulmonary surface lesions were present in four control calves and in each experimentally-infected calf. Pneumonic involvement was minimal in control calves (0.30 ± 0.35%) and congestion accounted for the majority of surface lesions in these calves. Pneumonic involvement was most extensive in Group II (15.15 ± 12.5%) and lobular consolidation accounted for the majority of surface pathology in these specimens. Nearly 30% of the pulmonary surface was pneumonic in two (118, 120) of the Group II calves. Pneumonic surface area involvement was similar in Groups III (6.5 ± 9.7%) and IV (4.0 ± 3.8%). Rechallenge exposure of Group IV calves did not increase the extent of pulmonary surface lesions. Significant differences in mean pneumonic surface area existed only between control and Group II calves.

Table 5: Pulmonary surface area measurements in experimental calves.

Group Calf #	Dorsal Surface: total area (cm^2)	Dorsal Surface: pneumonic area (cm^2)	Ventral Surface: total area (cm^2)	Ventral Surface: pneumonic area (cm_2)
Group I				
102	587.1	---	612.1	---
103	652.3	---	647.1	---
104	567.9	1.7	529.9	1.7
105	743.9	2.2	707.3	2.0
106	530.0	4.6	525.1	5.8
109	612.5	---	642.3	2.8
Group II				
115	569.7	9.2	489.5	8.3
116	456.2	50.3	404.5	54.3
117	477.9	3.6	481.2	6.9
118	628.8	132.9	573.2	222.4
119	589.2	96.7	598.9	114.3
120	548.5	146.3	534.6	164.1
Group III				
107	634.1	4.4	624.9	3.0
108	558.8	29.0	563.6	12.6
110	768.5	29.3	717.2	22.3
111	632.6	133.1	612.5	191.5
113	460.2	6.5	440.7	4.4
114	569.8	8.2	568.0	36.7
Group IV				
121	826.5	59.4	773.9	78.6
122	632.3	44.6	559.8	46.4
123	570.4	9.2	555.5	18.6
124	791.1	529.9	786.7	582.9
125	625.1	1.6	578.0	3.6
126	669.2	1.6	664.9	12.4

Table 6: Pneumonic proportions (%) of the total pulmonary surface area.

Group I	Group II	Group III	Group IV
Calf	Calf	Calf	Calf
102 - 0%	115 - 1.66%	107 - 0.58%	121 - 8.62%
103 - 0%	116 - 12.15%	108 - 3.71%	122 - 7.60%
104 - 0.32%	117 - 1.09%	110 - 3.46%	123 - 2.47%
105 - 0.29%	118 - 29.56%	111 - 26.07%	124 - 70.50%*
106 - 0.98%	119 - 17.77%	113 - 1.20%	125 - 0.43%
109 - 0.22%	120 - 28.66%	114 - 3.94%	126 - 1.05%
X ± SD = 0.30 ± 3.5%	15.15 ± 12.5%**	6.5 ± 9.7%	4.0 ± 3.8%

* This calf developed fatal bronchopneumonia and thus was excluded from data analysis and statistical consideration.

** Indicates significant difference ($P < 0.05$) between control and treatment group.

E. Histopathologic Examination

In Group I (controls), histologic lesions were not observed in the respiratory tract of three calves (102, 103, 106)(Figure 15). In the remaining three animals (104, 105, 109), mild inflammatory lesions were occasionally observed in sections of lung tissue. Approximately ten percent of the lung in calf 104 was affected by a mild bronchointerstitial pneumonia. Most of these changes, however, probably represented a response to proteinaceous foreign material (plant debris) observed within the lungs. In calves 105 and 109, focal sections of lung were characterized by mild lymphomononuclear infiltrates into peribronchiolar connective tissue (Figure 16). Inflammatory infiltrates often extended into surrounding alveolar lumens and into the interstitium of adjacent alveolar septa. A few alveoli contained scattered conglomerates of neutrophils (Figure 17). These changes may have represented an inflammatory response to the intratracheal sham inoculation.

A subacute, suppurative to pyogranulomatous, multifocally extensive, moderate to severe, bronchiolar interstitial pneumonia was the predominant histopathologic finding in Group II calves. Sections of trachea and bronchi appeared relatively normal (Figure 18). Severe lesions were restricted to the lungs. Many bronchioles contained intraluminal accumulations of fibrinocellular and mixed cellular (neutrophils, macrophages) exudates (Figure 19). Bronchiolar epithelium was generally hyperplastic with occasional mitotic figures (Figure 20). Peribronchiolar and perivascular areas were heavily infiltrated with lymphocytes, plasma cells, and macrophages (Figure 21). In one calf (120), rare epithelial and giant cell syncytia were observed in the lumens of affected bronchioles. Pathologic changes consistent with bronchiolitis obliterans were also

Figure 15: **Photomicrograph of a lung section from a Group I (control) calf. Alveolar sacs and terminal bronchioles are free of exudate. H and E stain. X 100.**

Figure 16: **Photomicrograph of a lung section from a Group I (control) calf. Mild lymphomononuclear infiltrate is evident in the interstitial tissue surrounding a terminal bronchiole. H and E stain. X 100.**

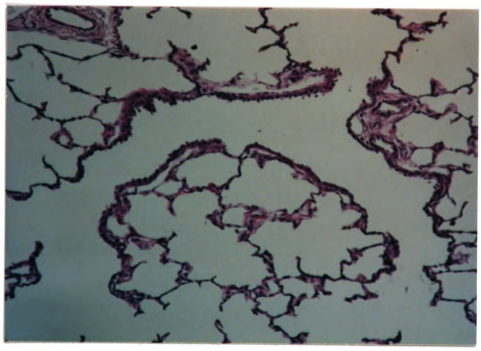

Figure 15

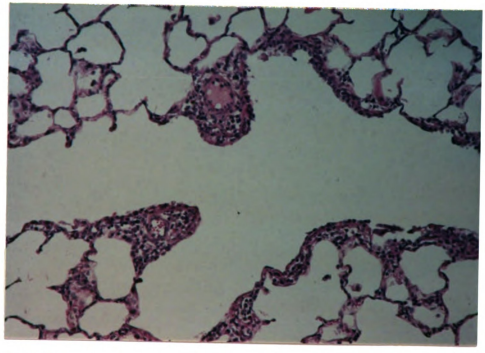

Figure 16

Figure 17: **Photomicrograph of a lung section from a Group I (control) calf showing a lymphomononuclear infiltrate in peribronchiolar interstitial tissue, alveolar septal thickening and mild, alveolar exudate. The alveolar exudate consisted of aggregates of neutrophils. H and E stain. X 100.**

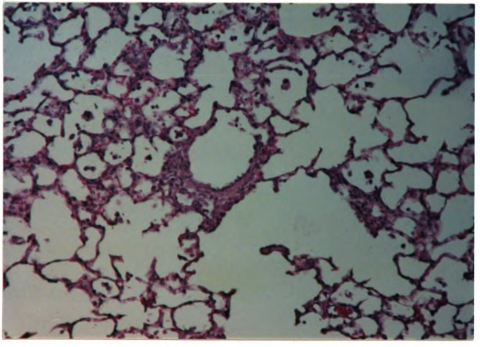

Figure 17

Figure 18: Photomicrograph of a small caliber bronchus from a Group II calf. Although there appears to be a mild increase in cellularity of the lamina propria, no inflammatory exudate is present within the lumen of this airway. H and E stain. X 40.

Figure 19: Photomicrograph of a terminal bronchiole from a Group II calf. The bronchiolar lumen is filled with a cellular exudate consisting of neutrophils and mononuclear cells. Peribronchiolar interstitial tissue contains increased lymphomononuclear cells. H and E stain. X 100.

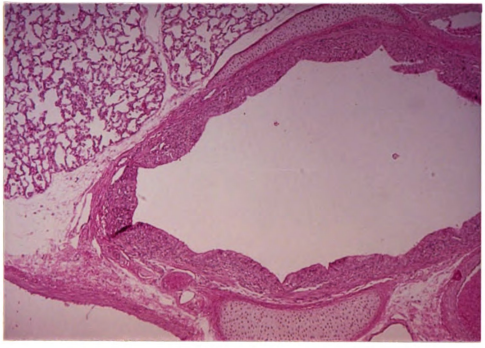

Figure 18

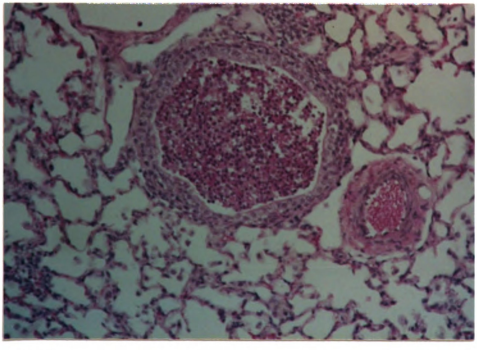

Figure 19

Figure 20: Photomicrograph of a terminal bronchiole from a Group II calf showing hyperplastic epithelium. Arrows point to mitotic figures commonly observed in the epithelium. H and E stain. X 250.

Figure 21: Photomicrograph of pulmonary parenchyma from a Group II calf showing peribronchiolar and perivascular infiltrate. This infiltrate consisted predominantly of macrophages, plasma cells, and lymphocytes. Exudate is evident in alveolar sacs. Alveolar septal walls are hypercellular. H and E stain. X 100.

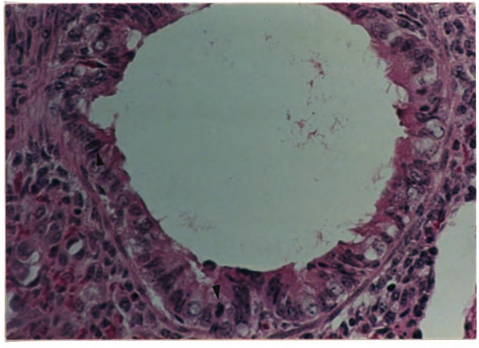

Figure 20

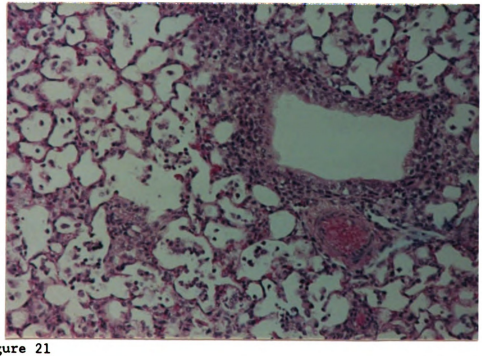

Figure 21

apparent in this same animal (Figure 22). Many alveolar lumens contained an inflammatory exudate of variable composition (neutrophils, lymphocytes, macrophages, sloughed alveolar epithelial cells, and hypereosinophilic fibrinous debris)(Figure 23). Alveolar walls appeared thickened due to atelectasis, edema, and lymphomononuclear infiltrates into alveolar septa (Figure 24). No alveolar epithelial syncytia or viral inclusion bodies were identified within this group. Multifocal areas of parenchymal abscessation were present in two calves (119, 120). Bronchial lymph nodes generally were markedly hyperplastic. In summary, severe microscopic lesions generally involved the terminal airways and alveolar sacs of Group II calves.

In Group III, histopathologic findings were characterized as a subacute, lymphomononuclear, multifocal, mild to severe bronchiolitis with an occasional accompanying broncho-interstitial pneumonia. In Group III, lesions were restricted to the lungs and primarily involved the bronchioles. Extensive lymphomononuclear peribronchiolar infiltration was the predominant lesion noted in these animals. In more severely affected regions of lung, the inflammatory reaction extended into adjacent bronchi, alveoli, and alveolar septa (Figure 25). Lymphocytic and mononuclear cells infiltrated the interstitial tissue (Figure 26) and scant accumulations of lymphocytes, neutrophils, macrophages, and rare giant cells were observed in alveolar and bronchiolar lumens (Figure 27). Most bronchial lymph node sections appeared markedly hyperplastic.

A subacute, nonsuppurative, diffuse, mild peribronchiolitis and bronchiolar-interstitial pneumonia characterized the histopathologic findings observed in Group IV. Accumulations of lymphomononuclear cells

Figure 22: **Photomicrograph of a bronchiole from a Group II calf showing bronchiolitis obliterans. Notice polypoid proliferation of granulation tissue into airway lumen. The epithelium overlying the granulation tissue is not intact. H and E stain. X 100.**

Figure 23: **Photomicrograph of a lung section from a Group II calf. Alveolar lumens contain a mixed cellular infiltrate consisting of neutrophils and mononuclear cells. Alveolar septal walls are hypercellular and capillaries are congested. H and E stain. X 250.**

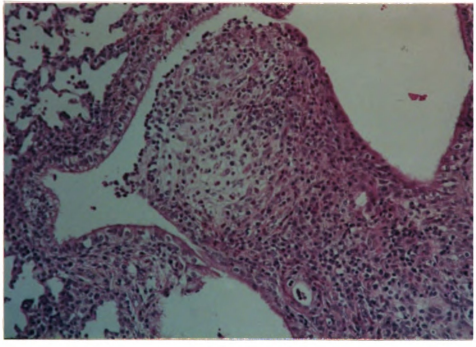

Figure 22

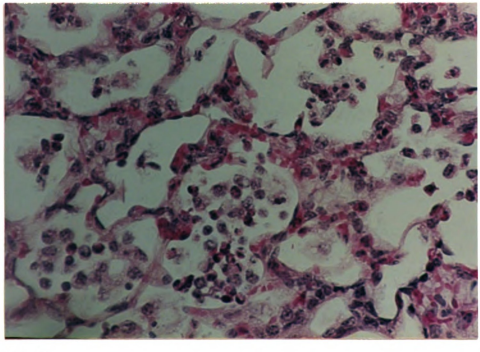

Figure 23

Figure 24: **Photomicrograph of a lung section from a Group II calf. Portions of the pulmonary parenchyma are atelectatic. Alveolar lumens contain eosinophilic fibrin tangles and inflammatory cells. Alveolar septal walls are hypercellular and individual septal walls are difficult to discern. H and E stain. X 250.**

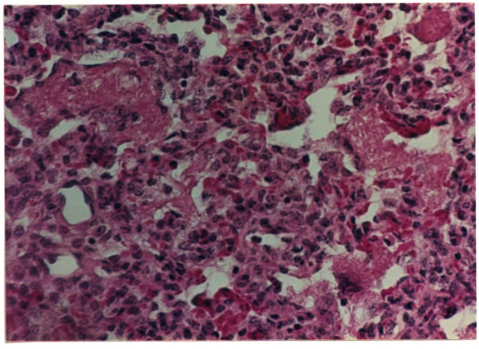

Figure 24

Figure 25: **Photomicrograph of a terminal bronchiole from a Group III calf. Notice moderate to severe lymphomononuclear infiltrate in the peribronchiolar interstitium. Adjacent alveolar septa are thickened and hypercellular. There is also increased cellularity in the adjacent alveolar sacs. H and E stain. X 100.**

Figure 26: **Photomicrograph of a terminal bronchiole from a Group III calf. A lymphomononuclear infiltrate predominates in the lamina propria and the peribronchiolar interstitium. A few scattered neutrophils are also present in these areas. H and E stain. X 250.**

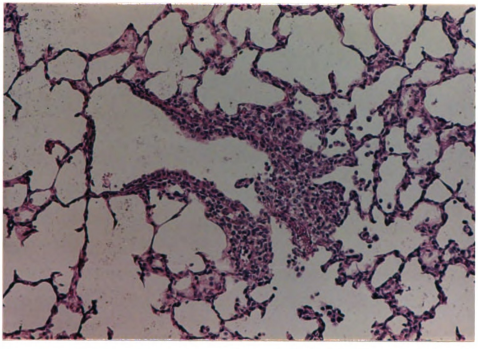

Figure 25

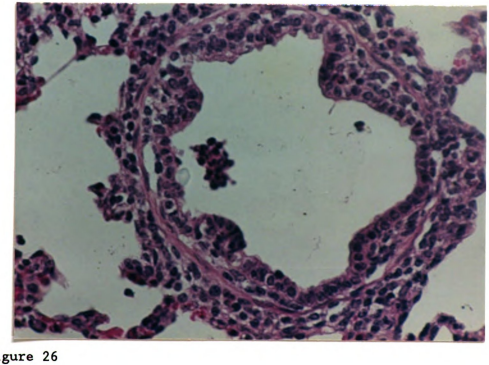

Figure 26

Figure 27: **Photomicrograph of a terminal bronchiole from a Group III calf. Fibrinocellular exudate fills the lumen of this airway. Notice syncytial giant cells (indicated by arrows) within this exudate. H and E stain. X 100.**

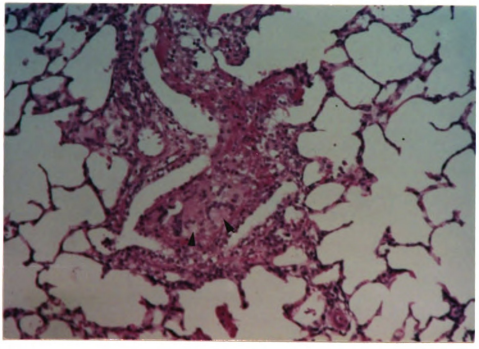

Figure 27

were visualized around bronchioles and adjacent blood vessels. The inflammatory cell infiltrates often extended for short distances from bronchioles into nearby alveolar septa and lumens. There was minimal bronchiolar and alveolar mucosal involvement, but syncytial cells were occasionally observed within bronchiolar and alveolar lumens. The syncytial cells were not epithelial in origin but were composed of mononuclear cells, giant cells and macrophages (Figures 28 and 29). In calf 124, bacterial invasion of the lower respiratory tract produced histologic lesions characteristic of severe bronchopneumonia with abscessation. However, a proliferative bronchiolitis, possibly associated with concurrent BRSV infection, was also observed in this case.

Figure 28: Photomicrograph (low magnification) of alveolar sacs from a Group IV calf showing cellular exudate in alveolar lumens. The cellular exudate consisted of neutrophils, macrophages, and syncytial giant cells. Arrows point to syncytial giant cells. H and E stain. X 100.

Figure 29: Photomicrograph (high magnification) of alveolar exudate with syncytial giant cells. H and E stain. X 250.

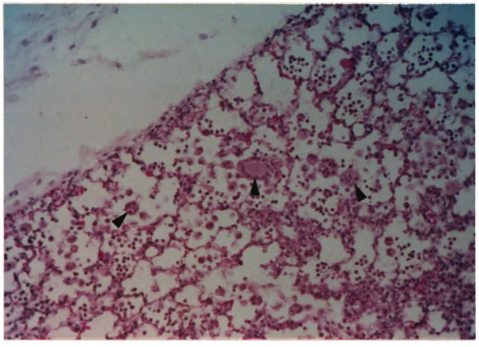

Figure 28

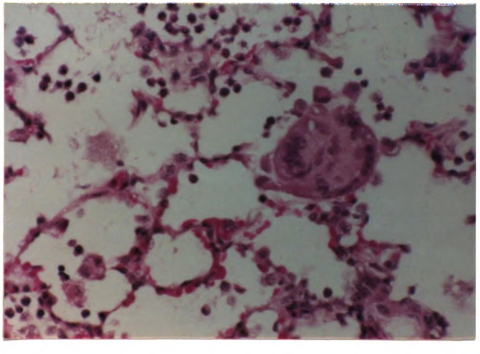

Figure 29

SUMMARY AND CONCLUSIONS

At least twenty different experimental attempts to reproduce respiratory tract disease with BRSV have been undertaken in calves and lambs during the past fifteen years. The majority of these studies have only resulted in mild disease with minimal respiratory tract lesions. A recent study (Bryson et al., 1982, 83; McNulty et al., 1983) was considerably more successful at reproducing respiratory tract disease and lesions in calves. However, the experimental subjects utilized in this research consisted of colostrum-deprived calves less than 7 days of age at the time of inoculation.

Although the inoculation protocol of Bryson (1982, 83) was adopted for the present study, experimental subjects were more representative of conventionally-raised calves. All calves received colostrum at birth and ranged between 3.5 and 4.5 weeks of age at the time of experimental infection. In the present study, severe respiratory tract disease and lesions were successfully induced in older, conventionally-reared calves utilizing a low-passage, field isolate of BRSV.

Biphasic respiratory tract disease has been observed in some natural BRSV disease outbreaks (Wellemans et al., 1978; Bohlender et al., 1982; Frey, 1982; Baker et al., 1986b). Biphasic disease associated with BRSV infection has been speculated to result from either 1) a persistent infection or 2) a hypersensitivity reaction to virus-infected cells (Bohlender et al., 1982; Frey, 1982; Frey, 1983). The present study was designed to determine if biphasic disease would result from a single in-

oculation in calves maintained for 24 days (group III) or if a re-exposure to the virus was needed to induce biphasic disease (group IV). A biphasic respiratory tract disease was not produced in either group III or IV. In natural BRSV outbreaks, biphasic disease may actually result from secondary bacterial or viral invasion rather than from a persistent infection or a viral hypersensitivity reaction.

Results of the current study also indicate that single exposure to BRSV, administered over four consecutive days, is capable of inducing severe respiratory tract disease and lesions in one-month-old calves. Re-challenge with this virus at 10 days after initial exposure did not cause exacerbation of clinical signs or respiratory tract lesions. In fact, group IV appeared resistant to BRSV infection at the time of second inoculation. Dual exposure of calves to BRSV has been previously attempted prior to the current study (Jacobs and Edington, 1975; Mohanty et al., 1976; Elazhary et al., 1981; Thomas et al., 1984b). As with the present study, rechallenge did not induce severe clinical signs or severe macroscopic lesions. However, all of these earlier investigations used an inoculation procedure that did not produce reliable results in terms of disease. Thus, when Bryson's inoculation protocol was shown to successfully produce disease and lesions, it was only logical to adopt his method to a rechallenge experiment.

Following viral inoculation of group III calves, experimental animals did not develop biphasic respiratory disease nor did they develop a persistent infection culminating in a hypersensitivity reaction to the virus. Viral rechallenge also failed to induce a viral hypersensitivity reaction in group IV calves. On the basis of clinical signs and experimental results, no indications were observed in this study to support a type I hypersensitivity reaction as the mechanism of BRSV pathogenesis.

Experimental infection induced clinical signs of respiratory tract disease in all principal groups. Viral reinoculation, however, failed to induce or accentuate clinical signs in group IV. The clinical signs observed during this study were very similar to signs described in naturally occurring BRSV outbreaks (Inaba et al., 1972; Bohlender et al., 1982; Verhoeff et al., 1984; Baker et al., 1986a, c). Anorexia, however, was not a part of the experimentally-induced clinical syndrome. In field outbreaks of BRSV, anorexia may be the result of secondary bacterial pneumonia. In this study, bacterial complications were not common in experimentally-infected calves.

Infection with BRSV produced several significant differences in clinical and clinicopathological responses between control and principal groups. Significant differences were observed in body temperature (Figures 1A and 1B), heart rate (Figure 2A), respiratory rate (Figures 3A and 3B), and arterial oxygen tension (Figure 4A). Although differences in heart rate and arterial oxygen tension were judged to be significant on days 1-8 (Figures 2A and 4A), this significance was not retained when groups were compared over the entire 24 day experimental period (Figures 2B and 4B). For both variables, measurements in principal calves returned to control levels after day 12. Inclusion of these normal data points in the statistical analysis cancelled the significance observed earlier in the experimental period.

Bovine respiratory syncytial virus infection did not produce significant differences in white blood cell count, differential leukocyte count, packed cell volume, plasma protein concentration, plasma fibrinogen concentration, and arterial carbon dioxide tension. Similar observations were made by Moteane (1978) in a previous study.

Sham inoculation of group I (control) calves produced minimal changes from baseline in body temperature (Figure 1B), heart rate (Figure 2B), and arterial oxygen tension (Figure 4B), but did not appear to influence respiratory rate (Figure 3B). The changes induced by sham inoculation possibly represented an inflammatory response of pulmonary tissue to the intratracheal administration of the sterile media. None of the changes observed in variables measured for the control group extended outside the accepted physiological range for young calves.

Rechallenge of group IV with BRSV had minimal influence on most clinical and clinicopathological responses. Rechallenge exposure did not exacerbate any of the observed changes in body temperature (Figure 1B), heart rate (Figure 2B), respiratory rate (Figure 3B), or arterial oxygen tension (Figure 4B) produced by the primary inoculation. In fact, heart rate and arterial oxygen tension returned to control levels in spite of BRSV reexposure. Rechallenge exposure, however, did appear to prolong the original elevations in body temperature and respiratory rate induced by the primary viral inoculation.

Bryson (1983) reported that rectal temperatures peaked at 39.4°C (102.9°F) in experimental calves following BRSV inoculation. In the current study, peak rectal temperatures of 40.6°C (105°F) were recorded in both group II and group III calves following inoculation.

Field observations indicated that passive immunity fails to protect calves from infection and disease caused by BRSV (Baker et al., 1986a). Also, it has been known for some time that experimental infection with BRSV is possible in calves despite the presence of passively-derived maternal antibodies (Smith et al., 1975; Jacobs and Edington, 1975; Mohanty et al., 1976). Recently, passive immunity has been shown

to totally suppress both local and systemic antibody responses following experimental infection of colostrum-fed, seropositive calves (Kimman et al., 1987). Seemingly, maternal antibodies fail to protect calves against BRSV infection and yet markedly inhibit the humoral response. In the present study, all experimental animals, except one (124), were found to possess maternally-derived serum antibody against BRSV. The presence of passive immunity failed to prevent BRSV infection and development of respiratory tract disease in calves utilized during this study. Graphic representation of geometric mean serum antibody titers (Figure 5) indicated that passive immunity effectively suppressed the systemic immune response. In this study, no calves seroconverted to BRSV following experimental infection. Viral rechallenge of group IV did not stimulate an anamnestic serum antibody response. However, BRSV rechallenge did not induce severe respiratory tract disease and lesions in these calves. Reinfection of colostrum-fed, seropositive calves has been shown to induce a protective, local immune response (IgA) within the respiratory tract (Kimman et al., 1987). Following reinfection, passive immunity apparently fails to inhibit the local immune response despite its continued suppression of the systemic humoral response (Kimman et al., 1987). The only calf (124) lacking passive immunity to BRSV in this research trial was also the only calf to develop fatal bronchopneumonia. The lack of passive immunity to BRSV in this calf may indicate a generalized failure or partial failure of passive transfer. Immunodeficiency may have increased this calf's susceptibility to secondary bacterial infection.

In the current study, gross lesions were produced in all experimentally-infected calves. All lesions were restricted to the lower respiratory tract and no gross lesions were found in other body systems. The

induced lesions closely resembled the gross changes produced experimentally by Bryson (1983) and were similar to the gross pathology described in many natural BRSV outbreaks (Pirie et al., 1981; Bohlender et al., 1982; Elazhary et al., 1982; Van Den Ingh et al., 1982; Baker et al., 1986c). Interstitial emphysema and mucopurulent bronchitis, frequently observed lesions in natural cases, were not common findings in experimentally-infected calves utilized in this study. Gross lesions were most extensive in group II. This group, however, was necropsied during the period of maximal clinical response. Group III and group IV had minimal lesions which appeared to be resolving at the time of postmortem examination. It is likely that gross lesions in groups III and IV were, at one time, comparable to the severe lesions observed in group II. Groups III and IV, however, were maintained for a longer period post-inoculation, thereby allowing time for resolution of pneumonic lesions. BRSV rechallenge did not increase the severity or extent of gross lesions in group IV. Group I (control) calves had exceedingly minimal gross changes.

In the present study, BRSV inoculation produced histopathologic lesions characteristic of a bronchiolar-interstitial pneumonia. Lymphomononuclear infiltrates were observed in bronchiolar lumens and peribronchiolar areas. In more severely affected calves, the inflammatory reaction usually extended for short distances into adjacent alveoli and alveolar septa. Syncytial cells, although rare in group II and group III, appeared commonly within bronchioles and alveoli in group IV. In general, the observed lesions closely resembled the microscopic pathology described in natural BRSV cases (Pirie et al., 1981; Van Den Ingh et al., 1982) and in previous experimental studies (Bryson et al., 1983). Bryson observed syncytial cells that were epithelial in origin while

Van Den Ingh commonly described syncytial giant cells. In the present study, syncytial giant cells and syncytial cells composed of macrophages and mononuclear cells were observed in pulmonary sections from group IV. In this study, intracytoplasmic eosinophilic inclusion bodies were never observed within syncytial cells as has been previously described (Pirie et al., 1981; Bryson et al., 1983). Histopathologic lesions were most extensive in group II. Although syncytial cells were most common in group IV, BRSV rechallenge did not increase the severity or extent of microscopic lesions in these calves. Intratracheal administration of the sterile inoculum produced mild inflammatory lesions in the lungs of three group I (control) calves.

Bacteria, mycoplasmas, and ureaplasmas were cultured, although infrequently, from the lungs and bronchial lymph nodes of some experimentally-infected calves. This finding is not surprising because respiratory viruses of cattle are believed to interfere with normal pulmonary clearance mechanisms, thereby allowing invasion by secondary pathogens (Dyer, 1982). The BRSV inoculum utilized in this study was prepared under aseptic conditions. Stock aliquots of fourth-passage virus were cultured and determined to be free of aerobic bacteria, Mycoplasma and Ureaplasma. Therefore, it is reasonable to assume that all bacterial, mycoplasmal, and ureaplasmal agents isolated from lung and lymph node specimens represent secondary invaders. It is known that these organisms can colonize the upper respiratory tract of healthy calves (Hamdy and Trapp, 1967; Corstvet et al., 1973). Similar pathogens have also been cultured from the lower respiratory tract in natural BRSV cases (Pirie et al., 1981; Baker et al., 1986c) and in previous experimental infectivity studies (McNulty et al., 1983; Castleman et al., 1985).

The contribution of the cultured agents to the observed disease and pathology was probably minimal. In this study, secondary pathogens were infrequently cultured. When demonstrated, they did not originate from many of the cases with the most severe clinical signs and respiratory tract lesions. In the current study, Pasteurella multocida was cultured from four calves and Pasteurella haemolytica was recovered from one calf. In natural BRSV outbreaks involving dairy calves, Pasteurella multocida was also the most commonly cultured bacterial pathogen (Baker et al., 1986c). Only one calf (124) in this study, however, developed a suppurative bronchopneumonia characteristic of Pasteurella multocida. Actinomyces pyogenes (Corynebacterium pyogenes), a bacteria commonly associated with abscessation and chronic pneumonias (Hjerpe, 1975), was recovered from two calves with pulmonary abscesses. Mycoplasmal and ureaplasmal agents were also cultured from two principal calves. Thirteen species of mycoplasmas and ureaplasmas have been isolated from the bovine respiratory tract (Gourlay et al., 1979), but their significance in bovine respiratory disease remains unclear. Only M. mycoides, M. dispar, M. bovis, and the ureaplasmas are considered to be of primary etiologic importance (Stalheim, 1983). Mycoplasma and Ureaplasma induced pneumonia is characterized by intense peribronchial lymphocytic hyperplasia which appears microscopically as a peribronchial cuff. A cuffing pneumonia was not observed in histologic sections from the two calves from which these agents were isolated.

Although histopathologic findings were highly suggestive of BRSV pneumonia, virus isolation conducted on nasopharyngeal swab samples was the only laboratory method to positively confirm BRSV infection in experimentally-infected calves. ELISA testing of nasopharyngeal samples,

virus isolation conducted on tissue specimens, and direct immunofluorescent testing of tissue samples all failed to demonstrate BRSV.

It is probable that nasopharyngeal swabs did not concentrate sufficient viral antigen to produce positive ELISA results. To optimize the ELISA procedure for diagnosis of HRSV in infants, the manufacturer strongly recommends a nasopharyngeal aspirate technique for specimen collection. Nasopharyngeal aspiration has been shown to be most effective in providing high quality specimens for these assays (ORTHO RSV Antigen, ELISA test, Ortho Diagnostic Systems, Raritan, NJ). Nasopharyngeal aspiration appears impractical in cattle and thus nasopharyngeal swabs were utilized in this study.

Virus isolation on fresh pulmonary specimens is currently not considered to be a reliable diagnostic technique (Baker and Frey, 1985; Baker et al., 1986c). Isolation of BRSV is usually successful only in the early phase of infection (Lehmkuhl et al., 1979). High levels of specific antibody in respiratory tract secretions and lung samples collected after the first or second day of disease may neutralize the virus and prevent isolation (Wellemans, 1977; Frey, 1983). In the present study, tissue specimens were collected several days to weeks after experimental infection (group II samples were collected on experimental day 9, group III and IV samples were collected on experimental day 24). In addition, if the virus is isolated in tissue culture, multiple subpassages in cell culture are generally required before the characteristic cytopathic effect is observed (Paccaud and Jacquier, 1970; Inaba et al., 1972; Smith et al., 1975; Lehmkuhl et al., 1979). In the current study, tissue samples were maintained in cell culture for 7 days.

Direct immunofluorescent examination of frozen lung sections has proven to be a successful method in the diagnosis of BRSV infections (Baker and Frey, 1985; Baker et al., 1986c). Precise reasons for the poor results obtained with direct immunofluorescence during the current study remain unknown. McNulty (1983) and Castleman (1985) both detected BRSV antigen in the lungs of calves necropsied between 2 and 10 days PI. In preparation for the present research trial, two pilot calves were experimentally-infected with the same BRSV isolate used in the study and positive immunofluorescence to BRSV was detected in the lungs of these calves using the same fluorescent antibody conjugate as in the study. Reasons for failure of the direct immunofluorescence technique may be similar to the reasons causing failure in virus isolation procedures (i.e. the presence of blocking antibody within the respiratory tract and timing of sample collection in relation to onset of infection). Other investigators have been unable to detect viral antigen with direct immunofluorescence during experimental BRSV infectivity studies in calves (Frey, M. Personal Communications, 1986).

In previous experiments, the extent of pulmonary lesions was based on an estimate of the percentage of lung surface with gross lesions (Al-Darraji et al., 1982a; Thomas et al., 1984a, 1984b; Trigo et al., 1984b). Estimation of pneumonic surface area is a highly subjective and variable procedure. The computer digitizing technique described in the present study, however, may represent the most standardized and reproducable method currently available for quantitation of pulmonary surface lesions. No previous studies have utilized this technique. Some limitations are still associated with this procedure. If the pneumonic surface area truly reflects the total extent of all pulmonary lesions,

it is assumed that all parenchymal lesions will correlate closely with the visible surface lesions. This may not always be a correct assumption. In the current study, one calf (121) had parenchymal lesions which were not visible on surface examination. Also, results of the computer digitizing technique are reported in terms of area (cm^2). Although a volumetric measurement would be most accurate, a volumetric measurement would be more difficult to obtain and would preclude submission of pulmonary samples for microbiological studies. Despite the described limitations, computer digitization is far superior to subjective estimation for documentation of pneumonic surface area. In the current study, surface lesions were most extensive in group II calves ($\bar{X}$ = 15.5%). It is possible that groups III and IV possessed, at one time, extensive surface lesions comparable to group II. Groups III and IV, however, were maintained for a longer period postinoculation, thereby allowing time for surface lesions to undergo resolution prior to necropsy. BRSV rechallenge of group IV failed to produce extensive surface lesions ($\bar{X}$ = 4.0%). Group I calves (control) had minimal pneumonic surface area involvement ($\bar{X}$ = 0.3%).

In conclusion, BRSV pneumonia resembling natural disease is difficult to consistently reproduce in experimental calves. In addition to BRSV exposure, several additional factors (environment, weather extremes, stress, weaning, transport, immune status, genetic composition, etc.) may be important in disease pathogenesis. Nonetheless, in this study, severe respiratory tract disease and lesions were successfully induced in one-month-old, colostrum-fed calves utilizing a low-passage, field isolate of BRSV. Single exposure to BRSV was found to be capable of inducing severe disease and respiratory tract lesions. Rechallenge with BRSV does not have a compounding or exacerbating effect. Severe pneumonic lesions were

observed in group II, whereas only mild lesions were present in groups III and IV. This finding suggests resolution of the viral pneumonia with time. Also, no experimental results or observations supported persistent infection or viral hypersensitivity as the pathogenetic mechanism of BRSV pneumonia. The field observation of biphasic respiratory tract disease in association with BRSV infection may be due to factors other than BRSV, such as secondary bacterial pneumonia.

Investigation of the potential synergistic relationship between BRSV and Pasteurella haemolytica and P. multocida within the bovine lung would appear to be a logical progression for future BRSV infectivity studies. Experimental inoculation of lambs with both agents has resulted in severe clinical disease and severe respiratory tract lesions (Al-Darraji et al, 1982a, b, c). The only calf (124) to develop fatal bronchopneumonia during the current project had experienced a concurrent bacterial invasion of the lower respiratory tract. Administration of BRSV in conjunction with Pasteurella haemolytica and P. multocida may be more effective in producing the severe disease and severe respiratory tract lesions commonly associated with natural outbreaks of calf pneumonia.

BIBLIOGRAPHY

BIBLIOGRAPHY

Al-Darraji AM, Cutlip RC, Lehmkuhl HD. Experimental infection of lambs with bovine respiratory syncytial virus and Pasteurella haemolytica: immunofluorescent and electron microscopic studies. Am J Vet Res 1982b; 43: 230-235.

Al-Darraji AM, Cutlip RC, Lehmkuhl HD, Graham DL. Experimental infection of lambs with bovine respiratory syncytial virus and Pasteurella haemolytica: pathology studies. Am J Vet Res 1982a; 43: 224-229.

Al-Darraji AM, Cutlip RC, Lehmkuhl HD, Graham DL, Kluge JP, Frank GH. Experimental infection of lambs with bovine respiratory syncytial virus and Pasteurella haemolytica: clinical and microbiologic studies. Am J Vet Res 1982c; 43: 236-240.

Bahnemann HG. Inactivation of viruses in serum with binary ethyleneimine. J Clin Microbiol 1976; 3: 209-210.

Baker JC. Studies on the bovine respiratory syncytial virus. Doctor of Philosophy Thesis. University of Minnesota, Minneapolis, MN, 1984.

Baker JC, Ames TR, Markham RJF. Seroepizootiologic study of bovine respiratory syncytial virus in a dairy herd. Am J Vet Res 1986a; 47: 240-245.

Baker JC, Ames TR, Werdin RE. Seroepizootiologic study of bovine respiratory syncytial virus in a beef herd. Am J Vet Res 1986b; 47: 246-253.

Baker JC, Frey ML. Bovine respiratory syncytial virus. Vet Clin North Am [Food Anim Pract] 1985; 1: 259-275.

Baker JC, Werdin RE, Ames TR, Markham RJF, Larson VL. Study on the etiologic role of bovine respiratory syncytial virus in pneumonia of dairy calves. J Am Vet Med Assoc 1986c; 189: 66-70.

Belshe RB, Richardson LS, London WT, Sly DL, Lorfeld JH, Camargo E, Prevar DA, Chanock, RM. Experimental respiratory syncytial virus infection of four species of primates. J Med Virol 1977; 1: 157-162.

Berthiaume L, Joncas J, Boulay G, Pavilanis V. Serological evidence of respiratory syncytial virus infection in sheep. Vet Rec 1973; 89: 337-338.

Bisgard GE, Ruiz AV, Grover RF, Will JA. Ventilatory control in the Hereford calf. J Appl Physiol 1973; 35: 220-226.

Bisgard GE, Vogel JHK. Hypoventilation and pulmonary hypertension in calves after carotid body excision. J Appl Physiol 1971; 31: 431-437.

Bohlender RE, McCune MW, Frey ML. Bovine respiratory syncytial virus infection. Mod Vet Prac 1982; 63: 613-618.

Bryson DG, McNulty MS, Allan GM, Cush PF, Logan EF. Experimental respiratory syncytial virus pneumonia in calves, in Proceedings. XII World Congress on Diseases of Cattle. World Association for Buiatrics, 1982; 66-71.

Bryson DG, McNulty MS, Logan EF, Cush PF. Respiratory syncytial virus pneumonia in young calves: clinical and pathologic findings. Am J Vet Res 1983; 44: 1648-1655.

Carbrey EA, Brown LN, Chow TL, Kahrs RF, McKercher DG, Smithers LK, Tamoglia TW. Recommended standard laboratory techniques for diagnosing infectious bovine rhinotracheitis, bovine virus diarrhea and shipping fever (Parainfluenza-3). Proc Annu Meet US Anim Health Assoc 1972; 75: 629-648.

Castleman WL, Chandler SK, Slauson DO. Experimental bovine respiratory syncytial virus infection in conventional calves: ultrastructural respiratory lesions. Am J Vet Res 1985b; 46: 554-560.

Castleman WL, Lay JC, Dubovi EJ, Slauson DO. Experimental bovine respiratory syncytial virus infection in conventional calves: light microscopic lesions, microbiology, and studies on lavaged lung cells. Am J Vet Res 1985a; 46: 547-553.

Chanock RM, Kapikian AZ, Mills J, Kim HW, Parrott RH. Influence of immunological factors in respiratory syncytial virus disease. Arch Environ Health 1970; 21: 347-355.

Chanock RM, Kim HW, Brandt CD, Parrott RH. Respiratory syncytial virus. In: Evans AS, ed. Viral Infections of Humans. New York: Plenum Medical Book Company, 1982; 471-489.

Chanock RM, Parrott RH, Vargosko AJ, Kapikian AZ, Knight V, Johnson KM. IV. Respiratory Syncytial Virus. Am J Public Health 1962; 52: 918-925.

Chanock R, Roizman B, Myers R. Recovery from infants with respiratory illness of a virus related to chimpanzee coryza agent. Am J Hyg 1957; 66: 281-290.

Coates HV, Chanock RM. Experimental infection with respiratory syncytial virus in several species of animals. Am J Hyg 1962; 76: 302-312.

Collier AM, Clydy WA. Model systems for studying the pathogenesis of infections causing bronchiolitis in man. Pediatr Res 1977; 11: 243-246.

Corstvet RE, Panciera RJ, Rinker HB, Starks BL, Howard C. Survey of tracheas of feedlot cattle for Haemophilus somnus and other selected bacteria. J Am Vet Med Assoc 1973; 163: 870-873.

Cutlip RC, Lehmkuhl HD. Lesions in lambs experimentally infected with bovine respiratory syncytial virus. Am J Vet Res 1979; 40: 1479-1482.

Doggett JE, Taylor-Robinson D, Gallop RGC. A study of an inhibitor in bovine serum active against respiratory syncytial virus. Archiv fur die gesamte Virusforschung 1968; 23: 126-137.

Donawick WJ, Baue AE. Blood gases, acid-base balance, and alveolar-arterial oxygen gradient in calves. Am J Vet Res 1968; 29: 561-567.

Dreizin RS, Vyshnevetskaia LO, Bagdamian EE, Yankevich OD, Tarasova LB, Kelenova AV. Study of experimental respiratory syncytial virus infection in cotton rats: virologic and immunologic studies. Vopr Virusol 1971; 16: 670-676.

Dyer RM. The bovine respiratory disease complex: a complex interaction of host, environment, and infectious factors. Comp Contin Ed Pract Vet 1982; 4: 296-307.

Elazhary MASY, Galina M, Roy RS, Fontaine M, Lamothe P. Experimental infection of calves with bovine respiratory syncytial virus (Quebec strain). Can J Comp Med 1980; 44: 390-395.

Elazhary MASY, Silim A, Morin M. A natural outbreak of bovine respiratory disease caused by bovine respiratory syncytial virus. Cornell Vet 1982; 72: 325-333.

Elazhary MASY, Silim A, Roy RS. Interferon, fluorescent antibody and neutralizing antibody responses in sera of calves inoculated with bovine respiratory syncytial virus. Am J Vet Res 1981; 42: 1378-1382.

Faden H, Kaul TN, Ogra PL. Activation of oxidative and arachidonic acid metabolism in neutrophils by respiratory syncytial virus antibody complexes: possible role in disease. J Infect Dis 1983; 148: 110-116.

Frey ML. Bovine respiratory syncytial virus and acute respiratory distress syndrome in cattle. Bovine Practitioner 1983; 18: 73-78.

Frey ML. The clinical significance of bovine respiratory syncytial virus, in Proceedings. Annu Fall Conf Vet University of Minnesota, 1982; 31-41.

Gourlay RN, Howard CJ, Thomas LH, Wyld SG. Pathogenicity of some Mycoplasma and Acholeplasma species in the lungs of gnotobiotic calves. Res Vet Sci 1979; 27: 233-237.

Hamdy AH, Trapp AL. Nasal microflora of feedlot calves before and after weaning. Am J Vet Res 1967; 28: 1019-1025.

Harrison LR, Pursell AR. An epizootic of respiratory syncytial virus infection in a dairy herd. J Am Vet Med Assoc 1985; 187: 716-720.

Hjerpe CA. Treatment of bacterial pneumonia in feedlot cattle, in Proceedings. 8th Annu Con Am Assoc Bov Pract 1975; 33-37.

Inaba Y, Tanaka Y, Omori T, Matumoto M. Physiocochemical properties of bovine respiratory syncytial virus. Jpn J Microbiol 1973; 17: 211-216.

Inaba Y, Tanaka Y, Sato K, Omori T, Matumoto M. Bovine respiratory syncytial virus studies on an outbreak in Japan, 1968-1969. Jpn J Microbiol 1972; 16: 373-383.

Jacobs JW, Edington N. Experimental infection of calves with respiratory syncytial virus. Res Vet Sci 1975; 18: 299-306.

Jain NC. Schalm's Veterinary Hematology. 4th ed. Philadelphia: Lea and Febiger, 1986.

Karber G. Beitrag zur kolletiven behandlung pharmakologischer reihenversuche. Arch Exp Path Pharmak 1931; 162: 480-481.

Kim HW, Chanchola JG, Brandt CD, Pyles G, Chanock RM, Jensen K, Parrott RH. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am J Epidemiol 1969; 89: 422-434.

Kimman TG, Westenbrink F, Schreuder BEC, Straves PJ. Local and systemic antibody response to bovine respiratory syncytial virus infection and reinfection in calves with and without maternal antibodies. J Clin Microbiol 1987; 25: 1097-1106.

Lea Master BR, Evermann JF, Mueller GM, Prieur MK, Vander Schalie J. Serologic and virologic studies on naturally occurring respiratory syncytial virus and Haemophilus somnus infections in sheep. Proc Annu Meet Am Assoc Vet Lab Diagn 1984; 26: 265-276.

Lehmkuhl HD, Cutlip RC. Experimentally induced respiratory syncytial viral infection in lambs. Am J Vet Res 1979a; 40: 512-514.

Lehmkuhl HD, Cutlip RC. Experimental respiratory syncytial virus infection in feeder-age lambs. Am J Vet Res 1979b; 40: 1729-1730.

Lehmkuhl HD, Gough PM, Reed DE. Characterization and identification of a bovine respiratory syncytial virus isolated from young calves. Am J Vet Res 1979; 40: 124-126.

McIntosh K, Fishaut JM. Immunopathologic mechanisms in lower respiratory tract disease of infants due to respiratory syncytial virus. Prog Med Virol 1980; 26: 94-118.

McNulty MS, Bryson DG, Allan GM. Experimental respiratory syncytial virus pneumonia in young calves: microbiologic and immunofluorescent findings. Am J Vet Res 1983; 44: 1656-1659.

Mohanty SB, Ingling AL, Lillie MG. Experimentally induced respiratory syncytial viral infections in calves. Am J Vet Res 1975; 36: 417-419.

Mohanty SB, Lillie MG, Ingling AL. Effect of serum and nasal neutralizing antibodies on bovine respiratory syncytial virus infection in calves. J Infect Dis 1976; 134: 409-413.

Morris JA, Blount RE, Savage RE. Recovery of a cytopathic agent from chimpanzees with coryza. Proc Soc Exp Biol Med 1956; 92: 544-549.

Moteane M, Babiuk LA, Schiefer B. Studies on the occurrence and significance of bovine respiratory syncytial virus in Saskatchewan. Can J Comp Med 1978; 42: 246-248.

Odegaard OA, Krogsrud J. A field outbreak caused by bovine respiratory syncytial virus. Acta Vet Scand 1977; 18: 429-431.

Paccaud MF, Jacquier CL. A respiratory syncytial virus of bovine origin. Archiv fur die gesamte Virusforschung 1970; 30: 327-342.

Pirie HM, Petrie L, Pringle CR, Allan EM, Kennedy GJ. Acute fatal pneumonia in calves due to respiratory syncytial virus. Vet Rec 1981; 108: 411-416.

Prince GA, Porter DD. The pathogenesis of respiratory syncytial virus infection in infant ferrets. Am J Pathol 1976; 82: 339-352.

Richardson LS, Belshe RB, London WT, Sly DL, Prevar DA, Camargo E, Chanock RM. Evaluation of five temperature-sensitive mutants of respiratory syncytial virus in primates. J Med Virol 1978b; 3: 91-100.

Richardson LS, Belshe RB, Sly DL, London WT, Prevar DA, Camargo E, Chanock RM. Experimental respiratory syncytial virus pneumonia in cebus monkeys. J Med Virol 1978a; 2: 45-59.

Rosenberger G. Clinical Examination of Cattle. 2nd ed. Philadelphia: W. B. Saunders Company, 1979; 73-75.

SAS Institute Inc. SAS User's Guide: Statistics. 5th ed. Cary, North Carolina: SAS Institute Inc., 1985; 114-137, 433-506.

Severinghaus JW. Blood gas concentrations. In: Fenn WO, Rahn H, eds. Handbook of Physiology, Respiration, Section 3. Vol 2, Washington DC: American Physiological Society, 1965; 1475-1487.

Smith MH, Frey ML, Dierks RE. Isolation, characterization and pathogenicity studies of a bovine respiratory syncytial virus. Arch Virol 1975; 47: 237-247.

Smith MH, Lehmkuhl HD, Phillips SM. Isolation and characterization of a respiratory syncytial virus from goats. Proc Annu Meet Am Assoc Vet Lab Diagn 1979; 21: 259-268.

Stalheim OHV. Mycoplasmal respiratory disease of ruminants: a review and update. J Am Med Assoc 1983; 182: 403-406.

Steel RGD, Torrie JH. Principles and Procedures of Statistics. 2nd ed. New York: McGraw-Hill Book Company, 1980; 377-398.

Stott EJ, Taylor G. Respiratory syncytial virus brief review. Arch Virol 1985; 84: 1-52.

Stott EJ, Thomas LH, Collins AP, Hamilton S, Jebbett J, Luther PD. The role of viruses in acute respiratory disease of cattle. In: Martin WB, ed. Respiratory Diseases in Cattle. Hague, Boston, London: Martinus Nijhoff, 1978; 230-240.

Taylor-Robinson D, Doggett JE. An assay method for respiratory syncytial virus. Br J Exp Pathol 1963; 44: 473-480.

Thomas LH, Stott EJ. Diagnosis of respiratory syncytial virus infection in the bovine respiratory tract by immunofluorescence. Vet Rec 1981; 108: 432-435.

Thomas LH, Stott EJ, Collins AP, Crouch S, Jebbett J. Infection of gnotobiotic calves with a bovine and human isolate of respiratory syncytial virus. Modification of the response by dexamethasone. Arch Virol 1984b; 79: 67-77.

Thomas LH, Stott EJ, Collins AP, Jebbett J. Experimental pneumonia in gnotobiotic calves produced by respiratory syncytial virus. Br J Exp Pathol 1984a; 65: 19-28.

Thomas LH, Stott EJ, Collins AP, Jebbett NJ, Stark AJ. Evaluation of respiratory disease in calves: comparison of disease response to different viruses. Res Vet Sci 1977; 23: 157-164.

Trigo FJ, Breeze RG, Evermann JF, Giallana AM. Pathogenesis of experimental bovine respiratory syncytial virus infection in sheep. Am J Vet Res 1984a; 45: 1663-1670.

Trigo FJ, Breeze RG, Liggitt HD, Evermann JF, Trigo E. Interaction of bovine respiratory syncytial virus and Pasteurella haemolytica in the ovine lung. Am J Vet Res 1984b; 45: 1671-1678.

Van Den Ingh T, Verhoeff J, Van Nieuwstadt A. Clinical and pathological observations on spontaneous bovine respiratory syncytial virus infections in calves. Res Vet Sci 1982; 33: 152-158.

Verhoeff J, Van der Ban M, Van Nieuwstadt A. Bovine respiratory syncytial virus infections in young dairy cattle: clinical and haemotological findings. Vet Rec 1984; 114: 9-12.

Waterman AE. Preliminary observations on the use of a combination of xylazine and ketamine hydrochloride in calves. Vet Rec 1981; 109: 464-467.

Wellemans G. Laboratory diagnosis methods for bovine respiratory syncytial virus. Vet Sci Commun 1977; 1: 179-189.

Wellemans G, Strobbe R, Van Opdenbosch E. The respiratory troubles of cattle in Belgium. In: Martin WB, ed. Respiratory Diseases in Cattle. Hague, Boston, London: Martinus Nijhoff, 1978; 248-256.

Welliver RC, Kaul TN, Ogra PL. The appearance of cell bound IgE in respiratory tract epithelium after respiratory syncytial virus infection. N Engl J Med 1980; 303: 1198-1202.

Welliver RC, Wong DT, Sun M. The development of respiratory syncytial virus specific IgE and the release of histamine in nasopharyngeal secretions after infection. N Engl J Med 1981; 305: 841-846.