Experimental combined infection of lambs with bovine respiratory syncytial virus and Pasteurella haemolytica

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Experimental combined infection of lambs with bovine respiratory syncytial virus and Pasteurella haemolytica

by

Ali Majeed Al-Darraji

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of The Requirements for the Degree of DOCTOR OF PHILOSOPHY

Major: Veterinary Pathology

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Pneumonia is one of the most common causes of death in feedlot and nursing lambs. No single etiological agent has been identified as a common denominator in lamb pneumonia. The disease appears to be caused by multiple infectious agents acting alone or in combination with non-infectious factors.

Infection with Pasteurella species is the most common cause of deaths among feedlot lambs in the U.S. and sheep in Britain. Many workers have been unable to reproduce the typical disease by inoculation of normal sheep with cultures of Pasteurella isolated from pneumonic lambs.

Recent work indicates that respiratory syncytial virus (RSV) is a respiratory pathogen in several species of animals both under natural and experimental conditions. The role played by RSV in respiratory disease of cattle is the most important among other known viruses in Belgium. Pneumonia and emphysema are associated with RSV infection in cattle.

Experimental infection of lambs with bovine RSV caused mild clinical signs and multifocal areas of interstitial pneumonia. Smith et al. found neutralizing antibodies to bovine RSV in sheep and in one study it has been shown that the prevalence of RSV complement-fixing antibodies in 31 tested ovine sera was high (81%) whereas they were relatively low in 28 bovine sera (14%) and in the sera of 34 equine (6%). The presence of RSV complement-fixing antibodies in sheep sera indicates that infection with RSV, either clinical or inapparent, has occurred. It remains to be determined whether or not RSV infection of lambs predisposes the respiratory system to infection by Pasteurella or other secondary pathogens.
The objectives of this study were to (1) investigate the role that RSV may play in preparing the ovine lung for invasion by Pasteurella haemolytica, (2) determine if there is an interaction between the two agents, and (3) more fully characterize and compare the lesions of experimental infection of lambs with RSV alone and in combination with Pasteurella haemolytica.

The experimentally infected lambs were studied clinically. Necropsies were performed and gross, microscopic, and ultrastructural changes were evaluated. Microbiological examinations included bacterial and viral isolation procedures, serologic studies, and localization of viral antigens in tissues by immunofluorescence.

This dissertation is presented in alternate format including three manuscripts to be submitted to the American Journal of Veterinary Research. All manuscripts are presented in the format required by the American Journal of Veterinary Research except footnote notation which conforms to standard thesis format. References cited in each manuscript are included with that manuscript in journal format. The manuscripts are preceded by a general introduction, objectives of the studies, and a critical review of the literature. General conclusions from the studies and acknowledgments follow the third manuscript. Additional literature cited refers to citations in the general introduction and is listed in the journal format consistent with that used in the manuscripts. The Ph.D. candidate, Ali M. Al-Darraji, was the principal investigator for each of the studies and is the senior author of each of the manuscripts.
LITERATURE REVIEW

Bovine Respiratory Syncytial Virus

**Historical aspects**

In 1956, Morris et al.\(^{14}\) first reported the isolation of an agent they suspected of causing an epizootic of respiratory disease in a colony of chimpanzees. The clinical signs of coryza produced by the virus led the investigators to term their isolate "the chimpanzee coryza agent." The disease syndrome involved only 20 animals; however, further studies with the virus have been extensive, expanding the field of both human and bovine respiratory disease research.

The first isolation from humans of agents indistinguishable from the chimpanzee coryza agent was in 1957 by Chanock et al.\(^{15}\) from children with severe lower respiratory disease. These two isolates, the Snyder and Long strains, along with the chimpanzee coryza agent, were grouped together as respiratory syncytial virus (RSV) based on their antigenic properties and the characteristic syncytium production in cell cultures. Further studies have implicated RSV as the single most important viral agent causing serious respiratory disease in infants and young children.\(^{16-18}\)

In 1968, Doggett et al.\(^{19}\) reported that the substances in bovine sera that inhibited the growth of the human respiratory viruses were actually neutralizing antibodies. Consequently, they postulated that cattle could be infected naturally with a virus yet to be isolated which should be antigenically related to human RSV.
In 1970, Paccaud and Jacquier\textsuperscript{20} first reported the isolation of RSV from cattle in Switzerland. Later in 1970, a bovine RSV that had been isolated in 1968 during an epizootic of so-called "cattle influenza" in Japan was described by Inaba et al.\textsuperscript{21} Wellemans et al.\textsuperscript{22} also reported the isolation of bovine RSV in 1970 during an outbreak in Belgium. Subsequent testing\textsuperscript{9,23} proved bovine RSV to be the most common agent in respiratory disease of cattle. In the U.S., Smith et al.\textsuperscript{24} reported the first isolation of bovine RSV. The isolate was obtained in 1971 from nasal secretions of Iowa feedlot calves with acute respiratory disease. They reported that 81% of 173 cattle from 43 herds in Iowa had serum antibodies to the bovine isolate.

Comparisons of human and bovine RSV with regard to antigenic, morphological, growth pattern, and pathogenetic characteristics have revealed some similarities as well as differences.\textsuperscript{25}

Smith et al.\textsuperscript{12} tested ovine sera against bovine RSV and reported that antibodies are also widespread in sheep. In a study in Canada,\textsuperscript{13} it was shown that the prevalence of RSV complement-fixing antibodies in sheep sera was high (81%) among 31 tested sheep, whereas they were relatively low (14% and 6%) in 28 bovine sera and in 34 equine sera respectively. The presence of RSV complement-fixing antibodies in sheep sera undoubtedly indicates a previous infection. Lehmkuhl et al.\textsuperscript{26} reported an increased rate of seroconversion for infectious bovine rhinotracheitis (IBR) virus, parainfluenza type 3 (PI-3) virus, bovine viral diarrhea (BVD) virus and bovine adenovirus type 3 in the presence of bovine RSV conversions. They suggested that bovine RSV may facilitate infection by other viruses.
The International Committee on the Taxonomy of Viruses has placed RSV in the genus *Pneumovirus* of the family *Paramyxoviridae*. This classification was based on the lack of serological relationship to other viruses of *Paramyxoviridae*, on the lack of hemagglutinin, hemolysin and hemadsorption activity and on the failure to grow in eggs.  

**Clinical signs**

Human RSV infections identifiable by virus isolation, are almost always symptomatic. Among older children and adults, the symptoms can be those of a "cold" confined to the nose and nasopharynx. In younger children and particularly in infants, lower respiratory tract involvement is the rule with a range from troublesome cough to severe respiratory distress due to bronchiolitis and or bronchopneumonia. The systemic response to the uncomplicated infection is not striking. Malaise is mild and the temperature is normal or slightly elevated. Neither atypical lymphocytes nor leukopenia characterize this virus infection.  

Morris et al.\(^{14}\) reported that RSV produces a clinical disease in chimpanzees that is characterized by coughing, sneezing, and mucopurulent discharge. Tyeryar et al.\(^{8}\) demonstrated that the chimpanzee was the only one of four species of primates to develop a clinical disease characterized by profuse rhinorrhea, mild cough, and sneezing.  

Infant ferrets are susceptible to infection with RSV in the upper and lower respiratory tract. But in young and adult ferrets there were no clinical changes observed.\(^{29}\) The cotton rat is also susceptible to RSV and the infection again is an anapparent one.\(^{30}\)
The cattle outbreaks in Japan that were caused by RSV were associated with an acute respiratory disease that was characterized by anorexia, depression, pyrexia, respiratory distress, cough, nasopharyngeal secretions, lacrimation, and foamy and sometimes blood-stained saliva. Among these outbreaks affecting 40,000 cattle, a 0.26% fatality was reported. Cattle outbreaks in Switzerland started suddenly and subsided after 3-5 days in calves and in 8-10 days in cows. Only cattle less than 7 years of age were clinically ill, with older affected animals having more frequent signs of bronchopneumonia than younger animals and no deaths or sequelae were reported.

In Belgium, infection with bovine RSV is suspected when all the cattle on the affected farm, especially "late calves," have a body temperature of about 40°C. The veterinarians treat affected cattle with antibiotics to combat secondary bacterial infection and so a drop in temperature occurs but 2-3 days later the animals experience difficulty in breathing and have bouts of dry cough. Respiration becomes increasingly rapid and shallow with little or no nasal discharge. There is frequent frothing at the commissures of the lips. Anorexia and constipation are common signs. On auscultation there is harshness in breathing but no rales. Emphysema noted at necropsy was considered a classic lesion of the disease and was usually seen in animals more than 4 months old. Up to 30% mortality was reported.

Jacobs and Edington inoculated gnotobiotic, colostrum-deprived, and conventional calves intranasally with 3 bovine isolates and 1 human
isolate of RSV. These experimental infections produced biphasic pyrexia associated with a serous nasal discharge.

Positive complement-fixing antibody tests were found to be associated with diverse disease problems in large production units of swine. Complement-fixing titers of 1:4 or above were also found in sera from lambs and swine in Illinois with the use of a human strain of RSV as antigen in the test. The complement-fixing seroconversions in lambs were not accompanied by any clinically detectable signs.

Experimentally, it has been shown that bovine RSV could infect lambs and cause a mild respiratory disease characterized by pyrexia evident on postinoculation (PI) days 3-9, hyperpnea, listlessness, hyperemic nasal mucosa and a slight increase in serous nasal discharge.

Gross pathology

In children, with the use of viral isolations and/or rise in antibody titer, RSV infection was found in 24% of those with pneumonia and in 40% of those with bronchiolitis. Descriptions of RSV lesions in the human literature are incomplete. Holzel et al. described the lung lesions in a boy with a clinical diagnosis of bronchiolitis from whom RSV was isolated prior to death. There were areas of emphysema, atelectasis and consolidation of the lung. Attempts to isolate the virus from human post mortem material have not been successful probably because the virus either was not present in the respiratory tract at the time of death or lost viability before necropsy samples were examined.
Transtracheal inoculation of $10^8$ plaque-forming units (PFU) of RSV into 14 cebus monkeys resulted in pneumonia in all 14. By PI day 2 there were areas of red consolidation throughout both lobes of the lungs and by PI day 8 there was gray consolidation. It was proposed that the cebus monkey is a suitable animal in which to study the pathogenesis of RSV pneumonia. 8

Paccaud and Jacquier 20 reported bronchopneumonia in cows infected with bovine RSV in Switzerland. Japanese cattle with fatal infections had emphysema, consolidation and edema of the lungs. 31 Wellemans 32 in Belgium reported widespread pulmonary emphysema in cattle that died of bovine RSV infection and the occasional occurrence of tracheitis or rhinitis.

Jacobs and Edington 33 experimentally infected cattle with three bovine isolates and one human isolate of RSV. Gnotobiotic, colostrum-deprived, and conventional calves were inoculated intranasally. The infection failed to produce macroscopic lesions. In another study, 36 small areas of lung consolidation in the right accessory and both cranial lobes were observed in calves. Wellemans and Leunen 9 compared the lesions in bovine RSV vaccinated and nonvaccinated cattle challenged with bovine RSV and reported localized pneumonia in nonvaccinated cattle which was much less severe lesion when compared to that seen in the vaccinated cattle. The authors pointed out the similarity of the bovine response to that of non-vaccinated and vaccinated humans to human RSV. 37, 38 Moteane et al. 25 experimentally infected one-week-old and seven-month-old calves with bovine RSV (Iowa strain) and reported no
macroscopic lesions.

Lehmkuhl and Cutlip\textsuperscript{39} experimentally infected feeder-age lambs with bovine RSV. The only lesion observed was petechiation in the apical and cardiac lung lobes of infected lambs on the third and fifth days PI. Inoculation of one-week-old lambs with RSV caused foci of hemorrhage and consolidation throughout all the lung lobes at 7 and 9 days PI.\textsuperscript{11} Consolidated areas had a pale-pink center and a red peripheral zone. Older consolidated foci (11 days PI) in the lungs were widely scattered and pale-pink to gray.

**Histopathology**

Reports on lesions in confirmed RSV infections in humans are rare.\textsuperscript{28} In children, structural pulmonary changes caused by RSV were of 2 kinds: (1) acute bronchiolitis, and (2) interstitial pneumonitis. Acute bronchiolitis is seen as epithelial necrosis and hyperplasia occasionally accompanied by intracytoplasmic inclusions in bronchiolar epithelial cells; later stages are characterized by bronchiolar plugging and peribronchiolar lymphoid infiltration. Hilar lymph nodes have moderate reactive hyperplasia.\textsuperscript{40} The other form, interstitial pneumonitis is characterized by a variable amount of airway epithelial destruction, thrombosis of fairly large pulmonary arterioles and interstitial or alveolar edema with hyaline membrane formation.\textsuperscript{40,41}

Holzel et al.\textsuperscript{35} described the findings in a 10-month-old boy with a clinical diagnosis of bronchiolitis from whom RSV was isolated 24 hours prior to death. Lesions of the bronchial mucosa consisted of
ballooning of the goblet cells and sloughing of the columnar layers of
the epithelium with infiltration of the bronchial wall by lymphocytes
and plasma cells; alveolar exudates consisted primarily of mononuclear
cells. There were neither multinucleated cells nor inclusion bodies.
However, Shedden and Emery\textsuperscript{42} saw multinucleated cells in the bronchial
epithelium of lung tissues from 4 children who died as a result of what
was described as "giant cell bronchitis." Their immunofluorescence
studies revealed an antigen of RSV or some closely related antigen in
the giant cells.

Ferrets intranasally inoculated with the A-1 strain of RSV had
destruction of nasal turbinate epithelium. The epithelial destruction
began on the third day PI and was most severe 6 days PI. Moreover,
multinucleated giant cells containing intracytoplasmic inclusion bodies
were present from the fifth to the ninth day PI. Thirty days PI, the
epithelium was back to the preinoculation state but the submucosa
appeared hypertrophied; the underlying cartilage was irregular in outline
and there were multinucleated cells in the perichondrium.\textsuperscript{43} Intranasal
inoculation of RSV into ferret kits resulted in degenerative rhinitis
but produced no definite pulmonary lesions.\textsuperscript{8} However, Coates and
Chanock\textsuperscript{43} reported occasional localized peribronchial and periarterial
lymphoid hyperplasia, giant cells throughout the lung tissue and foamy
macrophages in the alveolar spaces.

Henderson et al.\textsuperscript{44} compared the pathogenesis of human RSV infection
in organ cultures of 6-week-old ferret and fetal human origin tracheal
tissue. The patterns of virus growth were similar in the two cultures.
The virus replication in human organ cultures was limited to ciliated epithelial cells and these cells had ballooning degeneration and syncytium formation. In ferret cultures, virus was identified by immunofluorescence only in fibroblasts of the lamina propria and "serosa." There was no evidence of epithelial cell infection since there was no viral antigen present and histologically the tissue was normal.

Cell cultures infected with RSV characteristically form syncytia in which the nuclei are usually clustered centrally. After staining with Geimsa's or hematoxylin-eosin, eosinophilic cytoplasmic inclusions that are surrounded by a clear halo are found. Armstrong et al. described the patterns of cytopathic changes in Hela cell cultures inoculated with the Long strain of RSV. These consisted of multinucleated cells detectable after 24 hours, and by 48 hours the syncytia were large and numerous. The cell sheets then rapidly disintegrated. However, Jordan and Norrby et al. in their in vitro studies observed that the degree to which syncytia formation occurred was variable and was dependent upon the cell culture used.

It was shown that transtracheal inoculation of $10^8$ PFU of RSV into cebus monkeys resulted in histologic changes within 24 hours of inoculation. These changes consisted of thickening of the alveolar walls and filling of alveolar lumina with polymorphonuclear leukocytes and mononuclear cells. By day 4 PI there was extensive interstitial infiltration and giant cell formation in the alveolar wall. "Eosinophilic
inclusion bodies" were observed by day 6 PI but their specific location within the cell, or indeed the affected cell type were not reported. Extensive interstitial thickening, consolidation and fluid-filled alveoli persisted through PI day eight.

Bovine and human isolates of RSV given intranasally to gnotobiotic, colostrum-deprived and conventional calves produced only microscopic lesions characterized by focal degenerative rhinitis and catarrhal bronchiolitis. Occasional syncytia appeared to be free in the lumen of bronchioles and alveoli or were adherent to the bronchiolar epithelium. In one gnotobiotic calf given a bovine isolate (Dorset isolate), some of the focally-degenerating as well as normal epithelial cells of the nasal mucosa had intracytoplasmic inclusion bodies which stained postively with phloxine. Another bovine isolate (Hertford isolate) produced no lesions in the nasal mucosa oconventional or gnotobiotic calves, but did produce bronchitis in the conventional calves.

Bovine RSV infection resulted in variable degrees of pneumonia in calves. Interstitial pneumonitis with multinucleated giant cells and neutrophils in alveolar lumina was reported along with bronchitis and peribronchiolitis with focal squamous metaplasia of bronchiolar epithelium. The most severe changes were characterized by large peribronchial lymphocytic aggregates and masses of monocytes. Cortical and medullary edema, along with lymphocytic and reticuloendothelial hyperplasia were observed in bronchial lymph nodes. The above findings were in contrast to those of experimental infection of calves with bovine RSV
(Iowa strain) which produced only mild clinical disease and no gross or microscopic lesions.\textsuperscript{25}

Cutlip and Lehmkuhl\textsuperscript{11} experimentally infected lambs with bovine RSV and reported multifocal interstitial pneumonia and consolidation characterized by sloughing of epithelial cells of the airways and accumulation of necrotic debris, macrophages and neutrophils in the terminal airways and alveoli. Alveolar septa were infiltrated with numerous macrophages and lymphocytes. The alveolar lining was hyperplastic, and an occasional multinucleated cell was seen in alveoli or in respiratory epithelium. There was no evidence of inclusion bodies.

**Electron microscopic examination**

In a study of RSV morphogenesis in a monkey kidney cell line, no major nuclear change was reported but a few cells had slight chromatin margination. There were many compact intracytoplasmic inclusions with a granular to "thread-like" composition and occasionally free filamentous structures identical to those in the inclusions. There was also marked accumulation of glycogen in the cytoplasm.\textsuperscript{49} Armstrong et al.\textsuperscript{47} using Hela cell cultures, classified the cytoplasmic inclusions as two types. One was small and paranuclear with a fine fibrillar matrix and the other was larger, more widely distributed in the cytoplasm, and well demarcated with a dense amorphous or granular matrix. Both types were seen in syncytia but only the fibrillar-type inclusions were seen in mononuclear cells. Virus particles were observed only in the cell membrane.
The same workers\textsuperscript{47,49} also reported intranuclear inclusions in infected Hela cells. It had been reported\textsuperscript{47} that the inclusions were of the fibrillar type and suggested that they were of nucleolar origin because of their close relationship to the nucleolus and because the latter had regions of the same nature located in small cavities within it. They reported large and bizarre nucleoli as a feature of infected cell cultures. These workers also observed much folding of the cell membranes of mononuclear and syncytial cells in culture.

Henderson et al.\textsuperscript{44} studied 6-week-old ferret and fetal human tracheal cultures infected with RSV. In ferret tracheal cultures, fibroblasts were the only cells in which the virus replicated. The cells were studded with budding forms of RSV and had dense "rail-road track" membranes and large, regularly-spaced nucleoprotein cores. Cells had finely granular perinuclear inclusions. In contrast, in human fetal tracheal cultures, virus replicated only in the ciliated epithelium.

Cutlip and Lehmkuhl\textsuperscript{11} experimentally infected lambs with bovine RSV and found macrophages, neutrophils and necrotic debris in alveoli and airways. Granular pneumocytes were in excess and an occasional multinucleated epithelial cell was seen. Viral buds occurred on epithelial cells, and clusters of the virus were free within alveoli and bronchioles. There were also dense, granular, intracytoplasmic and mostly paranuclear inclusions in the epithelial cells.

Norrby et al.\textsuperscript{49} studied virion maturation in monkey kidney cells and reported that the process occurred at the cytoplasmic membrane or
at membranes of intracytoplasmic vesicles. In both cases, the membranes were thickened and short fringe-like structures were added to the membranes at the site where a cytoplasmic inclusion contacted the vesicle membrane or where a virion was budding from the cytoplasmic membrane.

Norrby et al.\textsuperscript{49} studied the structure of RSV and reported that complete virus particles were pleomorphic with a diameter of 90 to 130 nm, and had internal components consisting of 12 "dots" in radially symmetric arrangement. Some virus particles were elongated, with a uniform diameter of about 100 to 130 nm and a length occasionally exceeding 2 \( \mu \)m. Armstrong et al.\textsuperscript{47} estimated the particle diameter to be 65 nm. The above features of the virus are compatible with those of a myxovirus.\textsuperscript{47,49} Ito et al.\textsuperscript{50} studied the bovine RSV in calf kidney cell cultures and reported that in negatively-stained preparations the virus was pleomorphic and that many particles were roughly spherical with a diameter of 80 to 450 nm. In ultrathin sections the size of mature budding virus particle was 80-130 nm, and the internal components (the "dots" reported by Norrby et al.\textsuperscript{49}) varied from 11-15 nm. Based upon the structural similarities of bovine RSV to both human RSV and pneumonia virus of mice, Ito et al.\textsuperscript{50} proposed classifying all three agents within a common genus of the family Paramyxoviridae to be called Pneumovirus.

Cutlip and Lehmkuhl\textsuperscript{11} also reported that bovine RSV virions were pleomorphic. Filamentous forms, often with distended ends, had a dense outer coat covered with short projections and a series of inner dense
fibrils. Electron microscopic examination of negatively-stained bovine RSV revealed the virion to be highly pleomorphic with club-like surface projections. Free strands of nucleic acid were observed in the cell culture medium. They were filamentous, loosely arranged helices with the appearance of groups of disjointed rings.

According to the Paramyxovirus Study Group of the Vertebrate Virus Subcommittee of the International Committee on Taxonomy of Viruses, the family Paramyxoviridae contains 3 genera: Paramyxovirus, Morbillivirus and Pneumovirus. The genus Pneumovirus includes the respiratory syncytial viruses and the pneumonia virus of mice. Describing RSV structure, they stated that it has an envelope 7-15 nm thick covered with projections 10-12 nm long. The overall shape is pleomorphic, usually roughly spherical but filamentous forms are frequent. Pleomorphic and round forms are 80-500 nm in diameter, and the filamentous forms are 60-110 nm in diameter and up to 5 μm in length.

Immunologic-mediated RSV disease

Bovine RSV is antigenically closely related to, but distinct from a human RSV. Human RSV has been reported to be associated with allergic reactions in the lung of infants and children. In humans, individuals who have undergone infection often possess neutralizing antibodies in both serum and nasal secretion and there is a rough positive correlation between the levels of these substances.

One interesting aspect of the pathogenesis of the disease caused by respiratory syncytial viruses is that infection can occur in the presence of circulating antibodies. It has been suggested that
antibody may exacerbate the disease and be associated with allergic reactions characterized by asthmatic bronchitis in infants and children.\textsuperscript{37,53,54,57,58}

Chanock et al.\textsuperscript{59} have theorized that the presence of neutralizing antibodies in serum without the presence of protective IgA secretory antibodies in the respiratory tract is to be blamed for the allergic reaction. During infection of the lower respiratory tract, edema of the bronchiolar mucosa and alveolar wall allows the leakage of globulins through vascular walls. They suggest that the specific antibodies complex with viral antigens in the tissues to produce an immune complex (type 3) allergic reaction.

There are several bases for the above theory proposed by Chanock and his co-workers. These bases were drawn from: First, severe disease in children occurred in primary infections during the first few months of life and the timing correlated positively with the retention of passive serum antibodies (acquired maternally).\textsuperscript{59,60} This suggested that anti-RSV antibodies, which were widespread in the general population\textsuperscript{16} did not protect the infants. Second, the allergic reaction occurred in vaccine trials using parenteral inoculation of a killed cell culture vaccine which induced only serum neutralizing antibodies. Third, the vaccine experience had demonstrated that the peak age incidence of severe illness could be shifted from 1-3 months to 12-16 months if immune factors (i.e., serum antibody) were artificially stimulated in the latter age group. In one nursery, a naturally-occurring RSV outbreak took place 9 months after vaccination and revealed that: (1) the
frequency of viral isolation did not differ between vaccinees and controls, indicating that serum antibodies provided no protection against infection; (2) 60% of vaccinees developed bronchiolitis and/or pneumonia as compared with 8% of the non-vaccinated control children; and (3) a higher proportion of vaccinated (65%) than unvaccinated (0%) required hospitalization.

The idea of immune complex formation leading to lung injury was based on nontransferability of reaginic antibodies (needed for anaphylaxis), of lymphocytes (needed for cell-mediated immune response), and of IgM and IgA from mother to infant. Immunoglobulin G, however, is transferred to the offspring via the human placenta. It is not yet established whether IgG binds viral antigens on cell surface or binds the soluble antigens at extracellular sites. Should one or both of these reactions take place, complement would be expected to be bound also. However, complement levels were not observed to fall in the ill infants and complement fractions were not demonstrable in lung tissues post mortem.

In conclusion, 4 prerequisites are needed for immunologic disease to develop due to RSV: (1) virus must produce specific antigen at cell surface and/or excess soluble antigens in the respiratory tract epithelium; (2) specific protective IgA antibodies must be absent from respiratory secretions; (3) serum antibodies, either of maternal origin or vaccine-induced, must be present; and (4) the infant must be infected during early infancy (especially during 1-2 months of age).
Workers in Belgium\textsuperscript{9} compared bovine RSV to human RSV while attempting to develop a killed vaccine to bovine RSV. They reported that although vaccinated calves did seroconvert and were afebrile post-challenge, they developed more severe pneumonia than did the non-vaccinated calves. They concluded: (1) that further attempts to develop a killed bovine RSV vaccine should be abandoned, (2) that their results were proof that cattle react to bovine RSV in the same manner that humans do to human RSV\textsuperscript{37,38} and (3) that circulating antibodies cannot neutralize the virus in situ, and probably their presence causes an allergic reaction more severe than the virus caused inflammatory lesion.

The theory of an immune-mediated lung lesion related to RSV infection has not been tested in any other animal.

\textbf{PASTEURIELLA HAEMOLYTICA}

\textbf{Historical aspects}

\textit{P. haemolytica} is a member of the so-called "haemorrhagic septicemia" group of \textit{Pasteurella} organisms. It was first described by Jones\textsuperscript{62} in 1921. He isolated it from cattle and referred to it as \textit{Pasteurella boviseptica} group 1. Newsom and Cross\textsuperscript{63} studied cultures of \textit{Pasteurella} isolated from sheep and found that one group had identical biochemical and serological reactions to the Jones group 1. The name \textit{P. haemolytica} was proposed for this group because of a characteristic hemolysis on blood agar and failure to produce indole. There are two biotypes of \textit{P. haemolytica}, A and T, differing in their relative abilities to utilize arabinose and trehalose. They also differed in
colonial appearance, growth rates and pathogenicity. Biberstein et al. using an indirect hemagglutination technique based on soluble surface antigens, detected 10 serotypes of \textit{P. haemolytica}. The number has now been increased to 12 serotypes. Strains which are not yet serotyped can be isolated from healthy sheep as well as from those with pasteurellosis.

Smith reported that type A strains were associated with enzootic pneumonia of lambs and sheep and were almost always the cause of septicemic disease of lambs within the first few weeks of life. Cases of septicemia in older lambs were due to type T infections. In experimental infection in several laboratory animal species, morbidity and mortality rates were influenced by the strain of \textit{P. haemolytica} used. \textit{P. haemolytica} of biotype A is the organism most frequently associated with acute pneumonia in sheep in Britain. Thompson et al. serotyped 406 strains of \textit{P. haemolytica} isolated from cases of pneumonia pasteurellosis in sheep of all ages and reported that 58% were biotype A. They also indicated that serotypes A-1, A-2 and A-6 are probably responsible for most cases of ovine pneumonia pasteurellosis and that serotype A-2 comprised approximately one-third of the total.

Shreeve and Thompson reported that the first typable \textit{P. haemolytica} were detected in nasal swabs from lambs 48 hours after birth and that the number of serotypes carried by individual animals increased with age. The authors suggested that intimate and prolonged contact with their dams may facilitate the transfer of \textit{P. haemolytica} from the dam's more established respiratory flora.
Even though *P. haemolytica* is commonly isolated from the lungs of sheep with respiratory disease, attempts to produce disease with this organism alone have not been consistently successful.\(^72-74\)

*Pasteurella haemolytica* has been reported as the cause of fatal disease in sheep and cattle in the United States, \(^75,76\) Scotland, \(^77\) Canada, \(^78\) and Belgium. \(^79\) In the United Kingdom, available information suggests that pneumonia of sheep is of considerable economic importance.\(^2\) Infection with *P. haemolytica* species has been reported as the most common cause of death among feedlot lambs in the U.S. and sheep in Britain.\(^1,2\)

**Clinical signs**

In so-called acute pneumonic pasteurellosis, the disease starts suddenly with sheep dying or being acutely ill with obvious respiratory disease characterized by coughing and accelerated and difficult respiration and occlonasal discharge. On auscultation of the thorax there is consolidation of the anteroventral lung fields, bronchitis and pleurisy. The sheep usually have pyrexia and anorexia.\(^2\) Ovine pasteurellosis is also described as an acute infectious disease of feedlot and nursing lambs. The disease has a complex causation including *P. haemolytica* and is characterized clinically by pyrexia, nasal discharge, dyspnea and depression.\(^1\) Marshall\(^68\) reported that guinea pigs, white rats and rabbits are refractory to infection with \(10^9\) viable *P. haemolytica*. Mice were generally resistant, but the use of large inocula (\(4.6 \times 10^6\) viable *P. haemolytica*) caused death of 4 out of 10 mice after 72 hours.
Biberstein et al. stated "the most impressive feature of attempts to experimentally produce pneumonic changes by means of bacterial infection was the phenomenal tolerance displayed by the ovine lung to the introduction of enormous numbers of bacteria by most exposure techniques." They reported that doses ranging from $1.5 \times 10^9$ to $1.2 \times 10^{10}$ viable *P. haemolytica* of both A and T biotypes given by tracheobronchial intubation caused, at most, a transient rise in temperature in two-thirds of the cases (13 adult sheep). Six animals that developed lung lesions remained free from severe signs of generalized illness. Many workers have been unable to produce typical pneumonic pasteurellosis by parenteral inoculation of normal sheep with *Pasteurella* isolated from pneumonic lambs.

Smith reported that adult sheep were highly resistant to infection with serotypes of *P. haemolytica* given intratracheally. In 1964, he reported contradictory results when he gave *P. haemolytica* biotype A intratracheally to adult sheep; fatal pulmonary infections resembling acute forms of enzootic pneumonia were seen after large doses, whereas smaller doses caused nonfatal infections. Boidin et al. and Hamdy and Pounden inoculated lambs intranasally and/or intratracheally with *Pasteurella* isolated from sheep with pneumonia; the lambs remained clinically normal. Smith experimentally infected 3-week-old lambs intraperitoneally with strains of *P. haemolytica* of both biotypes. He reported that strains of biotype A but not of biotype T had a considerable virulence for lambs as evidenced by mortality.
Gilmour et al.⁷⁴ exposed nine specific pathogen free (SPF) lambs by aerosol to $10^{4.8}$ viable units of \textit{P. haemolytica} serotype A-1. One lamb died on day 7 PI without prior illness. Three lambs had dyspnea, listlessness and inappetance between days 4-8 PI and five lambs had no clinical signs. Sharp et al.⁸⁰ experimentally infected SPF lambs with PI-3 virus and \textit{P. haemolytica} biotype A serotype 1. None of the four lambs which received \textit{P. haemolytica} ($10^{6.1}$ viable units) alone developed respiratory illness. Two lambs were slightly dull and hyporexic for 1-3 days and one lamb was febrile.

Biberstein et al.⁸¹ attempted experimental reproduction of pneumonia in lambs using \textit{P. haemolytica} serotype A-5 and PI-3 virus. They reported that the clinical response of lambs infected with PI-3 virus alone was minimal. The contrast between lambs with dual infections and those receiving \textit{P. haemolytica} alone was far less clear cut than that between lambs with dual infection and those receiving PI-3 virus alone. In the group given \textit{P. haemolytica} alone, one of two lambs given the highest dose ($36 \times 10^8$ cells of \textit{P. haemolytica}) was pyrexic 24 hours PI and succumbed to the infection on the third day, but the other lamb did not become ill. Lambs inoculated with one-half ($18 \times 10^8$ bacteria) or one-fifth ($7 \times 10^8$ bacteria) of the highest dose of \textit{Pasteurella} only developed pyrexia.

\textbf{Gross pathology}

In cattle, the syndrome called "shipping fever" is described as an acute infectious disease characterized by acute inflammation of the nasal
cavities, paranasal sinuses, larynx, pharynx, trachea and bronchi and, in fatal cases, by an acute fibrinous pneumonia with bronchitis. The disease is caused by the interaction of *P. haemolytica* or *P. multocida*, PI-3 virus and environmental stress agents. Carter\(^2\) studied 36 cases of shipping fever in cattle, ranging in age from 4 to 12 months, and reported the main lesions to be an acute bilateral bronchopneumonia. The inflammatory process was characteristic of severe bacterial infection and *P. haemolytica* was isolated from 15 cases but other organisms were also isolated. He concluded that the disease was a true pasteurellosis. However, attempts to produce lesions using the isolated organism failed. Friend et al.\(^4\) inoculated 3 groups of 4, four-month-old calves intratracheally with 20 ml of bacterial suspension containing \(6.7 \times 10^{11}\), \(5.5 \times 10^{11}\) or \(5.4 \times 10^8\) of *P. haemolytica*. Calves in the respective groups were killed 18 hours, 3 days and 7 days PI. The most extensive gross lesions were present in calves of the second group (inoculated with \(5.5 \times 10^{11}\) bacteria and killed 3 days PI). The lesions consisted of severe and extensive consolidation of the apical, cardiac, and diaphragmatic lobes of the lungs; fibrinous pleuritis, and interlobular septal separation by fibrin.

Gilmour\(^2\) described the lesions in outbreaks of enzootic pneumonia of sheep caused by *P. haemolytica* biotype A. In the acute cases, the most striking gross lesions were pleuritis and pericarditis. Often there was straw colored pleural fluid with fibrin clots. The lungs were enlarged and edematous and had large bright-red to purplish-red areas of solid consistency in the ventral parts.
Froth could be expressed from the cut surface. Older lesions were sharply demarcated, less extensive and darker red. Abscesses were present with a central necrotic core and fibrous tissue capsule surrounded by pale gray lung tissue. There were broad adhesions between parietal and visceral pleura. Many workers were unable to produce pneumonic lesions by inoculation of normal sheep with Pasteurella isolated from pneumonic lambs. Biberstein et al. attempted experimental production of pneumonia in adult sheep using large doses (1.5 X 10^9 to 1.2 X 10^10 viable organisms) of a number of serotypes of P. haemolytica of both biotypes. They concluded that the pathological response to the organisms was so erratic that little can be deduced from the results concerning the relation of strains, types, sources and perhaps even dosage to virulence. They also reported that the macroscopic features of lesions produced by P. haemolytica biotype A were indistinguishable from those caused by biotype T.

In field observations, death in young lambs, the majority of which were 3-4 weeks old, was due to generalized infection with P. haemolytica. The postmortem findings consisted of fibrinous pleuritis, pericarditis and pneumonia, with hemorrhages in the pleura, pericardium and epicardium. Occasionally, acute fibrinous peritonitis and many subcapsular, pin-point, white foci in the liver were seen. The above lesions were reproducible experimentally in young lambs. Pneumonia and generalized infection were produced by intratracheal inoculation with large doses of P. haemolytica biotype A isolated from
field cases. He also reported that lambs were more susceptible to infection by the intraperitoneal than by the intravenous route of inoculation.

Smith\textsuperscript{67,85} experimentally infected 3-week-old lambs intraperitoneally with strains of biotypes A and T and found A biotype strains to be highly virulent, causing fatal acute fibrinous peritonitis within 36 hours of inoculation. The peritoneal exudate ranged from 10-100 ml.

Gilmour et al.\textsuperscript{74} exposed nine SPF lambs by aerosol to $10^4.8$ viable cells of \textit{P. haemolytica} serotype A-1. One of four lambs died. The lungs of four lambs including the one that died were dark red and had solid areas with necrotic foci in the apical and diaphragmatic lobes. Lungs of the other lambs were free of lesions.

Biberstein et al.\textsuperscript{81} experimentally produced pneumonia in lambs using \textit{P. haemolytica} serotype A-5 alone or in combination with PI-3 virus. The lambs given \textit{P. haemolytica} alone had dark red consolidated areas, especially in the anterior lobes. There were linear collapsed areas, necrosis, serous effusions and fibrinous pleuritis. Lesions were not dose-dependent, even though some quantitative variations of the lesions were evident among the survivors of three groups given the highest dose (36 X $10^8$ viable bacteria) or one-half or one-fifth that amount. Sharp et al.\textsuperscript{81} experimentally infected SPF lambs with PI-3 virus and \textit{P. haemolytica} A-1 and reported that each of the four lambs which received $10^6.1$ viable cells of the bacteria alone had a single small lesion in one lobe of the lung.
Histopathology

Many workers were unable to produce pneumonic lesions by inoculation of normal sheep with Pasteurella isolated from pneumonic lambs. Biberstein et al. reported that P. haemolytica biotype A can produce a lung lesion in adult sheep identical with that seen in enzootic pneumonia and that the histopathological features of such lesions were not distinguishable from those caused by biotype T. Friend et al. inoculated 4-month-old calves intratracheally with a large dose of P. haemolytica A-1 and reported that the most extensive lesions were present 3 days PI. These consisted of areas of coagulative necrosis, purulent bronchitis and bronchopneumonia. Alveolar spaces contained fibrin, edema, neutrophils, macrophages and occasional syncytial giant cells. Lymphatic vessels were distended by fibrin and neutrophils and were commonly thrombosed. There were elongated, fusiform, darkly basophilic macrophages and mononuclear cells that had the appearance of streaming out of alveolar ducts and enclosing areas of coagulative necrosis. These cells took a whorled pattern in the alveolar spaces. The above cells have been described as the most characteristic feature of pneumonic pasteurellosis and have been called "oat cells". Such cells have been observed both in field cases of pneumonic pasteurellosis in sheep, as well as in experimentally produced pneumonia using P. haemolytica biotype A in conventional lambs and in SPF lambs.

Multifocal areas of coagulative necrosis were seen in the lungs of adult sheep, lambs, and calves experimentally infected with P. haemo-
lytica and in field cases of ovine enzootic pneumonia. Gilmour et al. exposed 9 SPF lambs to an aerosol of P. haemolytica serotype A-1 and reported that the histological findings in four lambs consisted of an acute, exudative, nonproliferative pneumonia with focal necrosis bordered by alveoli distended by whorls of cells with flattened, elongated, basophilic nuclei. Acute vasculitis, thrombophlebitis and thrombose lymphatics were a consistent finding in pneumonic and non-pneumonic areas of lungs.

In outbreaks of ovine pneumonic pasteurellosis caused by P. haemolytica biotype A, the peracute lung lesion was that of alveolar necrosis, with the alveolar spaces filled with fluid and bacteria. Interlobular edema and fibrin deposits were described. There was bronchiolitis with sloughing of bronchiolar mucosa. In sheep which survive the peracute phase, hyperplasia of the mucosa and hypertrophy of cells lining alveoli were reported. Peribronchiolar lymphoid hyperplasia has been described in older lesions of field cases of enzootic pneumonia of calves and lambs, and in experimentally produced pneumonia in calves using P. haemolytica. In the latter case, bronchiolitis obliterans was also described. In cattle and sheep, there was fibrous tissue replacement or encapsulation of the necrotic lung tissue.

VIRAL-BACTERIAL COMBINED INFECTION

Pneumonia of sheep, like that of other species, is regarded to be of complex causation. Alley and Clarke suggested that
"evidence available does not favor the view that viruses play a major role in the etiology of pneumonias in sheep" but recent investigations have shown that viruses can predispose lambs to bacterial pneumonia, although specific virus-induced lesions may not be seen post mortem. These viruses are adenoviruses, PI-3 virus, reoviruses, and bovine RSV.  

In lambs, experimental viral infection will allow a low, otherwise innocuous, dose of P. haemolytica to cause pneumonia. Workers at the Moredun Institute, Edinburgh, showed that acute pneumonic pasteurellosis can be induced in SPF lambs by PI-3 virus and P. haemolytica biotype A-1. It has, however, proven difficult to consistently produce the disease with either agent alone. 

Sharp et al. considered the disease produced in SPF lambs by the combination of PI-3 virus and P. haemolytica to accurately represent the clinical and pathological changes that occur in pneumonic pasteurellosis. However, there is inadequate evidence of the importance of the combination of the two agents in the field situation. Indeed, the combination of PI-3 virus and P. haemolytica cannot consistently induce illness and lesions in conventionally reared sheep. Biberstein et al. suggested that there was less difference in the severity of illness between lambs with dual PI-3 virus-P. haemolytica infection and P. haemolytica infection alone than there was between lambs with dual infection (PI-3-P. haemolytica) and PI-3 virus infection alone.
In man, it is generally accepted that viruses can initiate pneumonia, yet the major gross lesions are caused by secondary bacterial invaders.\textsuperscript{97} Regarding the nature of the interaction, it has been suggested that bacteria present in the upper respiratory tract of otherwise healthy animals are able to proliferate in the lung following viral infection.\textsuperscript{89}

In lambs, PI-3 virus was reported to "enhance" the pathogenicity of \textit{P. haemolytica} A-1\textsuperscript{80,95} and that of other serotypes of \textit{P. haemolytica} as well.\textsuperscript{80} Indeed, their results indicate that the clinical signs and lesions of the illness produced by \textit{P. haemolytica} inoculated 7 days following virus infection were more severe than those produced by \textit{P. haemolytica} alone.\textsuperscript{80} It has been reported that infection of mice with parainfluenza type 1 virus (Sendai virus) impaired the clearance of \textit{Pasteurella pneumotropica} from the lower respiratory tract because of delayed intrapulmonary killing of the bacteria, enabling the latter to proliferate and cause pneumonia.\textsuperscript{98} There was a synergistic effect between the viral and bacterial infections, reaching a maximum when virus infection preceded bacterial infection by 6 days.

Jakab and Green\textsuperscript{99,100} studied the combined infection of Sendai virus and \textit{Staphylococcus aureus} in mice. They believed that impairment of the normal pulmonary clearance mechanism was due to failure of intracellular killing of bacteria by alveolar macrophages rather than to bronchiolar epithelial damage with delayed physical removal of bacteria due to a defect in the mucociliary stream or the alveolar transport system. Lopez et al.\textsuperscript{101} reported that PI-3 virus infection in
calves caused maximal impairment of normal bacterial clearance when *P. haemolytica* was inoculated 7 days after the virus. They saw no correlation between degree of virus-induced lesion to pulmonary retention of *P. haemolytica* and speculated that decreased clearance of *P. haemolytica* in lungs of calves 7 days PI of the virus was due to decreased phagocytic activity of alveolar macrophages.

The time that the bacterial infection was superimposed on the viral infection was crucial if maximum retention of bacteria was to result. In the combined infection of the murine respiratory tract with Sendai virus and *P. pneumotropica*, it appeared that the bacterial infection had to be superimposed 6-7 days after virus inoculation. This coincided with a decline in virus titers, the first detectable antibody response of the host, and a loss of viral inclusion bodies. Biberstein et al. in a detailed study of concurrent infection with PI-3 and *P. haemolytica* in sheep reported an increase in the severity of the infection occurred when large doses of *P. haemolytica* were inoculated 3 days after exposure to PI-3. These workers felt that there was no synergism between the two agents. Lopez et al. demonstrated that PI-3 virus infection in calves caused maximum impairment of normal bacterial clearance when *P. haemolytica* was inoculated 7 days after the virus.

Both complement-fixing antibody and neutralizing antibody to RSV have been found in sheep sera, indicating RSV's possible involvement as a respiratory tract pathogen in sheep. Bovine RSV was shown to infect lambs experimentally and to cause a mild clinical disease, suggesting that under certain environmental conditions or in combination
with other agents it could play a major role in the respiratory disease complex of sheep.11

Thomas et al.102 isolated both RSV and *P. haemolytica* and detected a serological response to each agent in 31 of 43 calves suffering an outbreak of respiratory disease. The clinical signs included pyrexia and hyperpnea. The authors stressed the striking temporal correlation of RSV and *P. haemolytica* infection in the calves since a comparable group of calves with a serological response to *P. haemolytica* but with no serological evidence of RSV infection, remained healthy. They stated that "The evidence neither reinforced nor discounted the possibility of interaction between RSV and *P. haemolytica* infection in the pathogenesis of the disease."

There are no published reports concerning combined infection of bovine RSV and *P. haemolytica* in sheep.
PART I: EXPERIMENTAL INFECTION OF LAMBS WITH
BOVINE RESPIRATORY SYNCYTIAL VIRUS AND PASTEURELLA HAEMOLYTICA --
CLINICAL AND MICROBIOLOGICAL FINDINGS

This manuscript will be submitted to the American Journal of Veterinary Research.
Summary

This report describes the clinical and microbiological features of experimental infection of 4-week-old lambs with a combination of respiratory syncytial virus (RSV) and Pasteurella haemolytica A-1. Twenty-two colostrum-deprived lambs were divided into 5 groups. Each group was given either sterile inocula or RSV and P. haemolytica alone or in combination. All lambs were injected transtracheally. When given in combination, RSV preceded P. haemolytica by 3 or 5 days.

In groups of lambs inoculated with P. haemolytica or RSV alone, a mild respiratory disease developed accompanied by a transient pyrexia in one and 2 lambs of the respective groups.

In 2 groups of the lambs inoculated with a combination of RSV and P. haemolytica, all lambs became listless and reluctant to move and some were recumbent or anorexic by 24 hrs post-inoculation of the bacteria (PIB). All had respiratory signs of hyperpnea and dyspnea and 10 of 11 lambs had pyrexia averaging 40.8 C. Some coughed and had serous nasal discharge. Signs persisted for 3 to 4 days and were more pronounced in those given bacteria 5 days after virus than in those given bacteria 3 days after virus.

Respiratory syncytial virus and P. haemolytica were isolated from 8 and 12 lambs respectively and from a total of 15 inoculated animals. A serologic response to RSV was detected in all lambs inoculated with the virus, but no antibody to P. haemolytica was detected.
Introduction

*Pasteurella haemolytica* is commonly isolated from field cases of respiratory disease in sheep. So called "pneumonic pasteurellosis" is described as an infectious disease of feedlot and nursing lambs that is characterized clinically by pyrexia, nasal discharge, dyspnea, and depression. It has been reported as a significant cause of economic loss in many parts of the world.\(^1\)\(^-\)\(^3\) Attempts to produce a comparable disease with *P. haemolytica* were not always successful.\(^4\)\(^-\)\(^8\) This is in agreement with the suggestion that as in other species, pneumonia of sheep is the result of complex etiologic factors.\(^9\)\(^-\)\(^11\)

Pneumonia in sheep appears to be caused by single or multiple infectious agents acting alone, in combination, or in concert with noninfectious factors. Among these organisms, *Pasteurella haemolytica* and parainfluenza type 3 (PI-3) virus have both been associated with ovine pneumonia.\(^1\)\(^,\)\(^4\)\(^,\)\(^12\)\(^,\)\(^13\) These two agents have been isolated from the same sheep with natural respiratory disease\(^9\)\(^,\)\(^14\) and experimentally they have produced an acute fibrinous pneumonia with necrosis when given together to specific pathogen-free (SPF) or conventional lambs.\(^15\)\(^-\)\(^17\)

Complement-fixing and neutralizing antibodies to respiratory syncytial virus (RSV) have been found in sheep sera and indicate possible involvement of RSV as a respiratory tract pathogen of sheep.\(^18\)\(^-\)\(^20\) There is no reference to the significance of the combined effect of RSV and *P. haemolytica* even though *P. haemolytica* can be isolated and a
serologic response to RSV can be detected\(^a\) in field outbreaks. Experimentally, bovine RSV was shown to infect colostrum-deprived lambs and to cause mild clinical disease characterized by pyrexia, hyperpnea, and listlessness. It was suggested that RSV, in combination with other agents or under certain environmental conditions, could play an important role in the respiratory disease complex of sheep.\(^21\)

The objectives of this study were (1) to determine if RSV infection would potentiate a Pasteurella infection and (2) to determine the critical time for the bacterial superimposition after the viral inoculation.

**Materials and Methods**

**Experimental Design** Twenty-two colostrum-deprived lambs were divided into five groups (Table 1). Groups 1 and 2 with 4 lambs each were given \(P.\) haemolytica and bovine RSV respectively. Groups 3 and 4 with 6 lambs each were given bovine RSV and 3 or 5 days later respectively were given \(P.\) haemolytica (one lamb of group 3 died of urethral obstruction and was not included in the study). A fifth group of 2 lambs was given sterile cell culture fluid followed in 3 days by bacterial culture fluid. All inocula, whether viral, bacterial, or culture fluids, were given by the transtracheal route. The lambs were observed

\(^a\)Lehmkuhl, H. D., unpublished data, 1980, National Animal Disease Center, P. O. Box 70, Ames, IA 50010
twice daily throughout the experiment for clinical signs of disease. Rectal temperatures were recorded daily, and nasal swabs were taken twice daily for bacterial and viral isolation.

Lambs were killed by being anesthetized with intravenous injection of sodium pentobarbital and succinylcholine chloride and exsanguinated by severance of the axillary vessels.

**Inocula**  The *P. haemolytica* used was biotype A serotype 1 (*P. haemolytica* A-1). It had been isolated from the lung of a feedlot calf that had died from acute respiratory disease and it was serotyped by the method of Frank and Wessman.\(^{22}\) To prepare the inoculum a small piece of frozen calf lung was streaked on blood agar base with 5% bovine blood. The plate was incubated overnight at 36°C. Three isolated colonies of *P. haemolytica* were selected and put into 150 ml of tryptose broth which was incubated while shaking for 18 hours at 36°C. This suspension of *P. haemolytica* was dispensed and frozen in 20 ml amounts. Before use, the *P. haemolytica* suspension was thawed and serial 10-fold dilutions were inoculated onto 5% bovine blood agar plates to determine the number of colony forming units (CFU) in the inoculum. The inocula for successive groups of lambs were prepared from the same frozen broth cultures. The viable cell count of the inoculum was \(3 \times 10^7\) CFU/ml and was constant after the initial freezing. Sterile tryptose broth was used to inoculate control lambs (group 5). Each lamb received 5 ml of the appropriate inoculum.

Isolate 232 of RSV was used as inoculum for the lambs. It was isolated from the lung of a lamb that had mild respiratory disease after
having been inoculated with a bovine isolate of RSV. The virus was grown in confluent ovine fetal kidney (OFK) cells maintained in Eagle's minimum essential medium (EMEM) supplemented with 5% fetal calf serum and 0.5% lactalbumin hydrolysate. When approximately 80% of the cells had evidence of virus-induced cytopathic effects (CPE) the cultures were frozen and thawed. Aliquots of the virus pool were frozen at -70°C until they were used for lamb inoculation. Each lamb received 20 ml of virus-containing tissue culture fluid, which contained 2.9 x 10^4 plaque forming units per ml. Noninoculated flasks of cells were processed in a similar fashion for inoculum for the control lambs (group 5).

Experimental animals Twenty-two, 4-week-old, colostrum deprived Columbia lambs were reared in isolation and fed commercial milk replacer. The lambs were observed for a period of one week before inoculation.

Isolation procedures Swabs of nasal secretions, tracheal fluids, and lung samples were collected for RSV isolation attempts. Specimens were processed within an hour after collection. Nasal and tracheal swabs were placed immediately in tubes containing 1 ml of EMEM supplemented with 2% bovine fetal serum, 0.1 mg of gentamicin sulfate and 10 μg of amphotericin B. The swabs were pressed against the side of the tubes. A portion of the swab suspension (0.25 ml) was inoculated into each of two 25 cm^2 tissue culture flasks containing 4 ml of a suspension of either ovine fetal lung (OFL) cells or OFK cells. Monolayers were formed within 24 hours. Lung samples were minced in
culture fluid and cocultivated with confluent monolayers of OFL or OFK cells. The monolayers were examined daily for 5 weeks for the presence of CPE and were subpassaged at intervals of 5 days.

Cultures of OFK and OFL cells were used since both have been shown to be suitable for RSV replication. The cells were passaged less than 10 times and grown as monolayers in EMEM supplemented with 10% ovine serum and gentamicin sulfate (0.1 mg/ml). Five percent irradiated bovine fetal serum was substituted for ovine serum for viral propagation and isolation attempts. Cell cultures were incubated at 37° C in a 5% CO₂ atmosphere and 98% humidity. Identification of the viral isolates was based on CPE and an indirect immunofluorescent test.

Portions of the dorsal and ventral aspects of the cranial part of the cranial lobe and of the caudal lobes were taken aseptically from the lungs in situ for bacterial isolation. These sites were chosen to see if viral infection would influence bacterial distribution within the lung. Specific sites were sampled from each animal without regard to lesion development at those sites. Specimens for bacterial culture were also collected from mediastinal lymph nodes, turbinates, trachea, spleen and blood. Routine cultural procedures were used. Attempts to isolate Haemophilus sp. and Mycoplasma sp. from the nasal cavity, trachea, and lung were also made. For the latter, a mycoplasma basal-supplement medium was used. Representative isolates of P. haemolytica were typed serologically using the rapid plate agglutination test.
Serology  Sera, collected before inoculation and at necropsy, were examined for antibody titers to bovine viral diarrhea (BVD) virus and to RSV using the microtiter serum virus neutralization test (SVN) previously described by Lehmkuhl and Gough.26

Attempts to detect a serologic response to *P. haemolytica* were made using the indirect haemagglutination test and the whole cell agglutination test.27

Results

Clinical signs  In group 1 (*P. haemolytica* alone), all 4 lambs appeared normal at 24 hours postinoculation of bacteria (PIB). One lamb was pyrexic on days 2, 3, and 4 PI (Fig. 1) and manifested reduced activity beginning on day 2. Two lambs had decreased physical activity and slightly accelerated respiration on day 3 PIB (Table 2). However, they were normal on the last day of the experiment (day 4 PI).

Infection of lambs with RSV alone was evaluated in group 2 and in groups 3 and 4 prior to bacterial inoculation (Table 3). Thus, clinical signs were observed in 15 lambs held for 3 days (groups 2, 3, and 4), in 10 lambs held for 5 days (groups 2 and 4) and in 2 lambs held for 7 days (group 2). All lambs were normal until day 3 postinoculation of virus (PIV) at which time there was slight to pronounced inactivity in 9 of the 15 lambs. An exception was one lamb in group 3 that had slight pyrexia on days 1 and 3 PIV (Fig. 2). On days 4 and 5 PIV, signs of decreased physical activity were more pronounced; more
lambs were involved, and there were respiratory signs and fever. On day 6 PIV, the 2 remaining lambs had no pyrexia (Fig. 3), and the other clinical signs were abating. On day 7 PIV, both lambs were clinically normal.

Clinical signs of illness in lambs of group 3 (RSV followed by \textit{P. haemolytica} in 3 days) and group 4 (RSV followed by \textit{P. haemolytica} in 5 days) were similar but were slightly more marked in the latter (table 4). By days 1 to 2 PIB all lambs were inactive and had respiratory illness. The cough was dry and intermittent and was especially evident when an animal was provoked; for example, nasal swabbing elicited paroxysms of coughing that persisted for up to 2 minutes. The respiratory rate averaged 52/minute and ranged from 38 to 72/minute.

In group 4, all lambs were pyrexic (>40.0 C) on days 1 and 2 PIB with temperatures averaging 41.4 C and 40.8 C respectively (Fig. 4). The temperature of each lamb of this group was higher at day 1 PIB than at day 2 PIB. In group 3, four and three lambs were pyrexic on days 1 and 2 PIB respectively. On these 2 days, their temperatures persisted essentially unchanged and were not as high as those of group 4.

In group 4, two lambs stood with their front legs apart and two others did not eat. These were the only anorexic lambs. In group 3, one lamb was observed shivering; this lamb had pyrexia throughout the experiment except on day 2 PIV. The lambs of both groups 3 and 4 had loss of condition by day 2 PIB which persisted to the end of the experiment. By day 3 PIB, the three lambs not killed in each of the two groups were clinically improved. However, in group 4, one lamb was
recumbent, and two still were dyspneic and hyperpneic; the next day (day 4 PIB) only one lamb had respiratory signs. In group 3, mild respiratory signs persisted in three and two lambs on days 3 and 4 PIB respectively.

Neither of the lambs in group 5 (controls) had pyrexia (Fig. 5) or clinical signs of disease throughout the experiment.

**Microbiology**  Respiratory syncytial virus was isolated from 8 of 15 lambs inoculated with virus alone or with virus plus *P. haemolytica* (Table 5). Virus was isolated from 2 of 4 lambs in group 2, from 4 of 5 lambs in group 3 and from 2 of 6 lambs in group 4. These isolations were from nasal secretions collected during the acute infection and from respiratory tissues collected at the time of necropsy. Respiratory syncytial virus was isolated less often from nasal swabs than from postmortem tracheal swabs and lung specimens.

Virus was not isolated from swabs or tissues taken from lambs in groups 1 and 5.

Early CPE of RSV in OFK and OFL cells consisted of granularity and shrinkage of a few cells in the culture. Within 4 days, the development of syncytial cells began by the coalescence of fewer than 10 cells which had considerable granularity to their cytoplasm. These syncytia developed a characteristic dirty yellow to brownish color. By 48 hours, especially in the case of lung samples grown on OFL cells, syncytia started to increase rapidly in number and size. In subsequent passages syncytia containing numerous nuclei floated free in the medium as progressively larger sheets, ultimately involving most of the monolayer.
In coverslip preparations stained with May-Grünwald-Giemsa stain the syncytia had nuclear aggregations toward their centers and large amounts of faintly eosinophilic cytoplasm. There were one or more dense, rounded, well-demarcated, eosinophilic cytoplasmic inclusions in the cells. Although CPE usually was evident in the first or second subpassage, it occasionally did not appear until the fifth subpassage.

The RSV that was isolated in cell cultures was identified by staining the viral antigens with specific antiserum using the indirect immunofluorescent test.

_Pasteurella haemolytica_ A-1 was recovered from 3 of 4 lambs in group 1, from all 5 lambs in group 3 and from 4 of 6 lambs in group 4 (Table 6). It was recovered from nasal swabs of 8 of 11 lambs of the combination groups (groups 3 and 4) and from 2 of 4 lambs of group 1. _Pasteurella haemolytica_ was isolated from the lungs and tracheas of 6 lambs and from the mediastinal lymph node and pericardial fluid of one lamb in groups 3 and 4. In group 1, _P. haemolytica_ was recovered from the lungs and tracheas of a total of 3 of 4 lambs. _Pasteurella haemolytica_ was not recovered from lambs of groups 2 and 5. All isolates were serologically homologous to that used in the inoculum.

_Escherichia coli_, _Klebsiella sp_, _Staphylococcus aureus_, _S. albus_, _Streptococcus haemolyticus_ (a and β), _Bacillus sp_, _P. haemolytica_ (not A-1) and _P. multocida_ were also isolated from the nasal passages on various days before and after inoculation. With the exception of _Bacillus sp_, which was isolated on one occasion from one lamb, the
other organisms were isolated from the nasal swabs of all 21 lambs examined. Attempts to isolate *Haemophilus* sp. or *Mycoplasma* sp. were not successful. The lungs and tracheas of lambs from groups 1, 3, and 4 either yielded a pure culture of *P. haemolytica* or were bacteriologically sterile. The lungs and tracheas of lambs from groups 2 and 5 were bacteriologically sterile. The spleens and blood of all lambs were sterile.

**Serology** The preinoculation SVN titers for RSV were negative (< 1:4) in lambs of all groups. All lambs of groups 2, 3 and 4 were RSV seropositive (>1:4) at necropsy. Following inoculation, there was a 2- to 7-fold or greater increase in titers of all lambs in groups 2, 3 and 4 (Table 7).

The postinoculation SVN titers for RSV in lambs of groups 1 and 5 were considered seronegative since they did not exceed 1:4.

Attempts to detect specific antibody to *P. haemolytica* were unsuccessful by whole cell agglutination and by indirect haemagglutination methods. No antibody to BVD virus was detected.

**Discussion**

Infection of lambs with *P. haemolytica* alone produced mild respiratory disease which persisted for only 24 hours in 2 of 4 lambs on day 3 PIB. One lamb had pyrexia from day 2 PIB until it was killed on day 4 PIB. These observations are compatible with those previously reported for *P. haemolytica* infection in lambs.8,14,15
In lambs inoculated with RSV alone, clinical signs of decreased physical activity were mild in 9 of the 15 lambs and pronounced in 2 of them. These signs progressed for 4 to 5 days PIV by which time respiratory signs were present. On day 7 PIV, the 2 surviving lambs were normal. The above signs were similar to those reported in lambs and calves inoculated with RSV. Although mild, the clinical signs seen in the lambs given RSV alone were more pronounced than those of lambs given *P. haemolytica* alone.

A combination of RSV and *P. haemolytica* produced a more severe clinical disease than did either agent alone. In lambs given RSV and *P. haemolytica*, respiratory signs and pyrexia were more severe, persisted for a longer period of time and involved a higher percentage of animals than in lambs given either agent alone. This was especially evident when a comparison was made with lambs given *P. haemolytica* alone. All lambs given both agents were dull and had respiratory signs (hyperpnea and dyspnea), and 10 of 11 lambs (90.9%) were pyrexic. Respiratory signs and pyrexia were generally more marked in lambs given *P. haemolytica* 5 days PIV than in those given *P. haemolytica* 3 days PIV. Although the clinical signs varied in degree and duration, the sequence of their appearance, progression and disappearance was similar in lambs with dual infection and in those infected with RSV alone.

Biberstein et al. reported that experimental combined infections of lambs with PI-3 virus and *P. haemolytica* resulted in more severe disease than that produced in lambs infected with either organism.
alone. The clinical response to PI-3 virus alone was minimal and in contrast to our findings with RSV, was less severe than the clinical response in lambs given *P. haemolytica* alone.

*Pasteurella haemolytica* A-1 was recovered from the lungs of a total of 6 of 11 lambs with dual infections and from the lungs of 2 of 4 lambs given *P. haemolytica* alone. The relatively low numbers of bacterial isolations from the lungs could be attributed to our system of sampling specific sites without regard to lesion development at those sites.

No conclusion could be drawn regarding differences in isolation of *P. haemolytica* from the 4 different locations of the lungs of lambs with dual infection. However, *P. haemolytica* was isolated 3 times from the dorsal portions of the caudal lobes and 4 times from the ventral portions of the caudal lobes of the lungs of lambs with dual infections. From these 2 sites, *P. haemolytica* was not isolated from the lungs of lambs given *P. haemolytica* alone. This finding indicates that RSV infection may have enhanced the distribution of *P. haemolytica*. Isolation of *P. haemolytica* A-1 from the mediastinal lymph node and pericardial fluid of one lamb given the two agents may similarly indicate the enhancement of the bacterial infection and early septicemia.

Bacteria (other than *P. haemolytica* A-1) isolated from nasal swabs were present in lambs of all 5 groups of this experiment and have been reported as part of the normal bacterial flora of the ovine upper respiratory tract.\(^1,33\)
Respiratory syncytial virus was isolated from the lungs of 8 of 15 lambs that had been inoculated with the virus but was not isolated from lambs inoculated with *P. haemolytica* alone or from the controls. There were no apparent differences in number of virus isolations from the 3 infected groups (2 with dual infections and 1 with RSV alone). The relatively few viral isolations from the nasal secretions lends further support to the suggestion that the primary site of viral replication is the lung. Most virus isolations from the lungs were obtained on days 5 and 7 PIV. This is different from previous reports that days 3 and 5 PIV in 6-month-old lambs and days 7 and 9 PIV in 1-week-old lambs were the days of most frequent viral isolations from the lungs. Note that in our experiment none of the experimental lambs were killed 3 days PIV and only 3 lambs in group 4 (RSV *P. haemolytica* in 5 days) were allowed to live 9 days PIV. These 3 lambs had a high antibody response, and thus the virus may have been neutralized by the time of euthanasia.

An antibody response to RSV occurred in all lambs inoculated with virus, and titers were higher in lambs that were killed at the end of the experiment than in those killed earlier. No lamb mounted an antibody response to *P. haemolytica* during the relatively short (2 to 4 days) intervals between inoculation and euthanasia. One of the tests used, namely whole cell agglutination, has been shown to give the best results with regard to measurement of serologic response of calves to *P. haemolytica*.27
The lesions observed in lambs with dual infections (to be reported elsewhere) were similar to those previously described\textsuperscript{5,34,35} in naturally occurring cases of acute pneumonic pasteurellosis. Therefore, on the basis of the clinical, microbiological and pathological findings, it is concluded that dual infection with RSV and P. haemolytica produces a more severe acute respiratory disease than that produced with either agent alone and that the severity of illness is a reflection of synergistic action of the two agents. It is also concluded that the most severe signs and lesions result when exposure to P. haemolytica occurs five days following exposure to RSV.
Table 1. Experimental Design - Temporal Relation of Inoculation and Euthanasia of Lambs with RSV and P. haemolytica

<table>
<thead>
<tr>
<th>No. of Day</th>
<th>Group</th>
<th>Animals</th>
<th>Day</th>
<th>Day</th>
<th>Day</th>
<th>Day</th>
<th>Day</th>
<th>Day</th>
<th>Day</th>
<th>Day</th>
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<tbody>
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<td>Kill 2</td>
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<td>P. haemolytica</td>
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<td>BCF§</td>
<td>Kill 2</td>
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*5 ml of bacterial inoculum with a viable cell count of $3 \times 10^7$ colony forming units/ml.
†20 ml of RSV inoculum with $2.9 \times 10^4$ plaque-forming units/ml.
‡20 ml of sterile cell culture fluid.
§5 ml of sterile bacterial culture fluid.
Table 2. Clinical Signs of Group 1 (*P. haemolytica* alone)

<table>
<thead>
<tr>
<th>Clinical Sign</th>
<th>0-2 days PI</th>
<th>3-4 days PI</th>
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<td>Reduced activity</td>
<td>1/4*</td>
<td>2/2</td>
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<tr>
<td>Listlessness</td>
<td></td>
<td>2/2</td>
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<tr>
<td>Reluctance to move</td>
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<td></td>
</tr>
<tr>
<td>Reluctance to stand</td>
<td></td>
<td></td>
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<tr>
<td>Nasal discharge</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperpnea</td>
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<td>2/2</td>
</tr>
<tr>
<td>Dyspnea</td>
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<td></td>
</tr>
<tr>
<td>Coughing</td>
<td></td>
<td></td>
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<tr>
<td>Loss of condition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrexia</td>
<td>1/4</td>
<td>1/2</td>
</tr>
</tbody>
</table>

PI = post inoculation

*Number lambs with sign/number at risk

- = not present in any animal
Table 3. Clinical Signs of Group 2 (RSV alone)

<table>
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<th>Clinical sign</th>
<th>0-3 days PI</th>
<th>4-5 days PI</th>
<th>6-7 days PI</th>
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<td>Reduced activity</td>
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<td>9/10</td>
<td>2/2</td>
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<tr>
<td>Listlessness</td>
<td>5/15</td>
<td>9/10</td>
<td>2/2</td>
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<td>Reluctance to move</td>
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<td>9/10</td>
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<td>Reluctance to stand</td>
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</tr>
<tr>
<td>Hyperpnea</td>
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<td>8/10</td>
<td>2/2</td>
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<td>Dyspnea</td>
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<td>7/10</td>
<td>2/2</td>
</tr>
<tr>
<td>Coughing</td>
<td>-</td>
<td>2/10</td>
<td>1/2</td>
</tr>
<tr>
<td>Loss of condition</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pyrexia</td>
<td>1/15</td>
<td>3/10</td>
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</table>

PI = post inoculation

*Number lambs with sign/number at risk

- = not present in any animal
Table 4. Clinical Signs of Group 3 (RSV Followed by *P. haemolytica* in 3 Days) and of Group 4 (RSV Followed by *P. haemolytica* in 5 Days)

<table>
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<tr>
<th>Clinical sign</th>
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<th>Group 4</th>
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<tr>
<td></td>
<td>0-2 days PIB</td>
<td>3-4 days PIB</td>
</tr>
<tr>
<td>Reduced activity</td>
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<td>3/3</td>
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<tr>
<td>Listlessness</td>
<td>5/5</td>
<td>3/3</td>
</tr>
<tr>
<td>Reluctance to move</td>
<td>3/5</td>
<td>2/3</td>
</tr>
<tr>
<td>Reluctance to stand</td>
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<td>-</td>
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<tr>
<td>Nasal discharge</td>
<td>3/5</td>
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<td>Hyperpnea</td>
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<td>Dyspnea</td>
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<tr>
<td>Coughing</td>
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<td>1/3</td>
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PIB = Post inoculation of the bacteria

*Number lambs with sign/number at risk

- = not present in any animal
Table 5. RSV Isolations from Nasal and Tracheal Swabs and from Lung Tissues of Lambs Inoculated with RSV Alone (Group 2) and with RSV and P. haemolytica (Groups 3 and 4)

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<th>4</th>
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<th>8</th>
<th>9</th>
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PIV = postinoculation of the virus
*Day of euthanasia
- = Negative for RSV
+ = Positive for RSV
Table 6. Sites of *P. haemolytica* Isolation from Lambs Experimentally Infected with RSV and *P. haemolytica* (Groups 3 and 4) and *P. haemolytica* Alone (Group 1)

<table>
<thead>
<tr>
<th>Group</th>
<th>Lamb #</th>
<th>Ante-mortem</th>
<th>Post-mortem</th>
<th>Lung</th>
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Abbreviations:
*Nasal swabs collected daily for 2 or 4 days depending on time of euthanasia*
Dcr = dorsal portion of the cranial part of the cranial lobe
Vcr = ventral portion of the cranial part of the cranial lobe
Dca = dorsal portion of the caudal lobe
Vca = ventral portion of the caudal lobe
MLN = mediastinal lymph node
+ or - = indicates the presence or absence of *P. haemolytica* A-1
Table 7. Serum Virus Neutralization Titers* of Lambs Inoculated with P. haemolytica Alone (Group 1), RSV Alone (Group 2), RSV and P. haemolytica (Groups 3 and 4) and Controls (Group 5)

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<th>Group</th>
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<td>1965</td>
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*Reciprocal of dilution

†Titers <1:4 are considered negative
Fig. 1. Temperature response of lambs inoculated with *P. haemolytica*. Average daily response of the lambs and their temperature range indicated by bar.
Fig. 2. Temperature response of lambs inoculated with RSV and *P. haemolytica*. Average daily response of lambs inoculated at day 0 with RSV and at day 3 with *P. haemolytica*. The temperature range is indicated by bar.
Fig. 3. Temperature response of lambs inoculated with RSV. Average daily response of the lambs and their temperature range indicated by bar.
Fig. 4. Temperature response of lambs inoculated with RSV and *P. haemolytica*. Average daily response of lambs inoculated at day 0 with RSV and at day 5 with *P. haemolytica*. The temperature range is indicated by bar.
Fig. 5. Temperature of lambs inoculated with sterile inocula. Average daily temperature of lambs inoculated at day 0 with sterile cell culture fluid and at day 3 with sterile bacterial fluid. Temperature range is indicated by bar.
References


PART II: PATHOLOGY OF EXPERIMENTAL COMBINED INFECTION
OF LAMBS WITH BOVINE RESPIRATORY SYNCYTIAL VIRUS
AND PASTEURELLA HAEMOLYTICA

This manuscript will be submitted to the American Journal of
Veterinary Research.
Summary

The pathology of experimental combined infection of 4-week-old lambs with respiratory syncytial virus (RSV) and Pasteurella haemolytica A-1 was studied. Twenty colostrum-deprived lambs were given RSV, P. haemolytica or a combination of the two. Inocula were administered transtracheally and in those lambs allocated to dual infection groups, inoculation with RSV preceded that of P. haemolytica by 3 or 5 days. A control group of two colostrum-deprived lambs was given a sterile inoculum transtracheally.

Lesions were seen in 2 of 4 lambs inoculated with P. haemolytica alone. Grossly, these were focal to diffuse pneumonic lesions with hemorrhagic and necrotic centers and fibrinous pleuritis. Histologically, the salient features were acute fibrinous pneumonia with necrosis of the lung parenchyma and fibrinous pleuritis.

In 3 of 4 lambs inoculated with RSV alone, gross lung lesions were multifocal areas of consolidation and hemorrhages. Histologically, these were characterized by interstitial pneumonitis, slight bronchiolitis and focal hemorrhages.

Pneumonic lesions were seen in all 11 lambs given both RSV and P. haemolytica. They were extensive in 6 of the lambs, were more severe than those in lambs given either agent alone, and were qualitatively similar to those in lambs given the bacteria alone. Lesions were most severe in lambs inoculated with bacteria 5 days rather than 3 days following inoculation with RSV. Histologically, the lesions were severe,
acute, diffuse, exudative pneumonia with focal necrosis and hemorrhages accompanied by acute fibrinous pleuritis.

Lesions seen in lambs given RSV and P. haemolytica in combination or in lambs given P. haemolytica alone were grossly and histologically like those of natural cases of acute pneumonic pasteurellosis.
Introduction

*Pasteurella haemolytica* is often isolated from sheep with pneumonia. The pneumonia associated with *P. haemolytica* has two principal forms: (1) an acute form (known as pasteurellosis, acute pneumonic pasteurellosis, acute necrotizing pneumonia, or acute necrotizing or exudative pneumonia),\(^1\)\(^-\)\(^5\) and (2) an atypical form (known as atypical or enzootic pneumonia).\(^3\)\(^,\)\(^5\)\(^-\)\(^9\)

Both forms of pneumonia were considered to be etiologically complex.\(^1\)\(^0\)\(^-\)\(^1\)\(^4\) *P. haemolytica* was associated with both forms,\(^3\)\(^,\)\(^5\) but experimental reproduction of the disease with this organism by either intratracheal or intranasal routes of inoculation has not been uniformly successful and requires very large doses of inocula.\(^4\)\(^,\)\(^1\)\(^5\)\(^-\)\(^1\)\(^9\) Therefore, it has been postulated that a predisposing agent is necessary for *P. haemolytica* to become established in the lungs of sheep and cause disease.\(^7\)\(^,\)\(^1\)\(^8\)

Pulmonary damage by a virus, for example, parainfluenza type 3 (PI-3) virus, may allow *P. haemolytica* to proliferate and cause a *Pasteurella*-type pneumonia in sheep.\(^1\)\(^9\)\(^-\)\(^2\)\(^1\) The infectious agents which have been reported as predisposing factors to *P. haemolytica* infection of the ovine lung include adenovirus,\(^2\)\(^2\) reovirus,\(^2\)\(^3\) respiratory syncytial virus (RSV),\(^2\)\(^4\) chlamydia,\(^1\)\(^8\) and mycoplasma\(^2\)\(^5\) in addition to PI-3 virus.

It has been reported that mild infection of the murine lung withSendai virus will cause failure of the intracellular killing of *Staphylococcus aureus* by alveolar macrophages, thus resulting in impairment of the pulmonary defense mechanisms. This allows for proliferation of
bacteria resulting in lung lesions more severe than those produced by
either agent alone.\textsuperscript{26,27} Lopez et al.\textsuperscript{28} reported that PI-3 infection in
calves caused maximum impairment of phagocytic elimination of \textit{P. haemo-
lytica} when the bacteria were inoculated 7 days after the virus.
Pulmonic lesions of the combined infection could not be evaluated
because the calves were killed 4 hours post inoculation of the bacteria
and not enough time had elapsed for pneumonic lesions to develop.

Respiratory syncytial virus is an important pathogen of the lower
respiratory tract in children\textsuperscript{29-31} and can infect lower primates,\textsuperscript{32,33}
sheep,\textsuperscript{34,35} and cattle.\textsuperscript{36,37} Bovine RSV infectivity in cattle was con­
firmed by its isolation from cattle with respiratory disease.\textsuperscript{38-42}
Smith et al.\textsuperscript{43} found neutralizing antibodies to bovine RSV in sheep sera
indicating previous exposure to the virus. In one study, the incidence
of RSV complement-fixing antibodies in 31 sheep sera was high (81%),
whereas it was relatively low (14\% and 6\%) in 28 bovine sera and in 34
equine sera respectively.\textsuperscript{44}

Experimentally, it has been shown that RSV could infect lambs and
cause mild clinical respiratory disease.\textsuperscript{24} Lesions were characterized
grossly by focal pulmonary hemorrhages and consolidation. Histologic
lesions were characterized by bronchitis and bronchiolitis with necrosis
of bronchial and bronchiolar epithelium and accumulation of necrotic
cellular debris leading to plugging of terminal airways and alveoli.
There were occasional multinucleated giant cells present in alveolar or
bronchial epithelium.
The lesions of natural cases of acute ovine pneumonic pasteurellosis have been described.\(^6,15,45,46\) It is generally characterized as an acute exudative necrotizing pneumonia.

The present study was designed to investigate the pathology of the combined intratracheal inoculation of lambs with RSV and \(P.\) \(\text{haemolytica}\) and to compare the results with lesions induced by each agent alone and with those of reported naturally-occurring cases of acute pneumonic pasteurellosis.

**Materials and Methods**

Details of the experimental procedures have been reported (Al-Darraji et al.\(^47\)). Briefly, twenty-two, four-week-old Columbia lambs were divided into 5 groups. Groups 1 and 2, with 4 lambs each, were inoculated with \(P.\) \(\text{haemolytica}\) or bovine RSV respectively. Groups 3 and 4 with 6 lambs each were inoculated with RSV and at 3 or 5 days post inoculation of the virus respectively were inoculated with \(P.\) \(\text{haemolytica}\). One lamb of group 3 died of urethral obstruction and was not included in the study. Group 5, with 2 lambs, was given sterile inocula of cell culture fluid and 3 days later sterile bacterial culture fluid. All the inocula were given transtracheally.

The lambs were colostrum-deprived, reared in isolation rooms and fed milk replacer throughout the experiment.

\(\text{Pasteurella haemolytica,}\) biotype A, serotype 1 (\(P.\) \(\text{haemolytica}\) A-1) was used. It was obtained from a feedlot calf that had died from acute
respiratory disease. Each lamb received 5 ml of an inoculum containing $3 \times 10^7$ viable cells/ml.

Respiratory syncytial virus isolate 232 was used as inoculum for the lambs. The virus was isolated from the lung of a lamb with mild respiratory disease after having been inoculated with a bovine isolate of RSV.$^{34}$ Each lamb received 20 ml of a tissue culture fluid that contained $2.9 \times 10^4$ plaque-forming units of virus per ml.

Postmortem examination The 22 lambs were killed according to the schedule outlined previously.$^{47}$ They were anesthetized with intravenous injection of sodium pentobarbital and succinyl choline and exsanguinated by severance of the axillary vessels.

With the lungs in situ, portions of the dorsal and ventral regions of the right caudal lobe and of the cranial part of the right cranial lobe were excised for histopathologic examination. The lungs were removed and examined for gross lesions. The excised lung and portions of lung lesions were fixed in 10% neutral phosphate-buffered formalin and Bouin's fixative. Pieces of the following tissues were collected in 10% neutral phosphate-buffered formalin for histopathologic examination: mediastinal lymph node, trachea, nasal turbinates, liver, spleen, kidney, heart, urinary bladder, stomach, duodenum, pancreas, ileum, mesenteric lymph node, spinal cord, and brain. After fixation, tissues were processed routinely for paraffin embedding, and 5 μm sections were stained with haematoxylin and eosin. Lung sections were also stained with periodic acid-Schiff (PAS), Van Geison's stain, Gram's stain, Macchialvello's stain and acridine orange stain.
Results

Gross lesions  The lungs of 2 lambs in group 1 (P. haemolytica alone) were free of lesions. Both lungs of a third lamb had areas of consolidation with central necrosis and hemorrhages both in the area of necrosis and peripheral to the area of consolidation. Two consolidated areas were seen in the central region of the cranial lobes. One area was quadrangular and 1 by 2 cm, and the other was round and occupied most of the central part of the cranial lobe of the left lung. The latter had a hemorrhagic, necrotic center, 1.5 cm in diameter (Fig. 1). There was also an area of consolidation in the antero-ventral portion of the caudal lobe of the right lung with scattered petechiae throughout all lobes of the right lung.

The fourth lamb from this group had hemorrhagic areas in the lobes of its right lung and the ventral part of the cranial lobe of the left lung had a well demarcated, roughly spherical consolidated lesion with a diameter of 3.5 cm (Fig. 2). There were fibrinous tags on the pleura of this part of the lung with adhesion to the pericardial sac; there was also slight hydropericardium.

In 3 of 4 lambs of group 2 (RSV alone), there were areas of consolidation scattered throughout the lungs. These areas were slightly raised, pale pink, had well-delineated borders and were surrounded by emphysematous tissue. They were usually discrete and less than 0.5 cm in diameter, but in one lamb the lesion involved most of the proximal third of the caudal lobe of the right lung (Fig. 3). The lesions were more
frequent in the caudal lobe but were also seen in the cranial and middle lobes. Petechiae or ecchymoses less than 0.5 cm in diameter were seen in all lung lobes and were especially common in the cranial and caudal lobes. Foci of atelectasis associated with the above described lesions were present. One lamb of group 2 was free of lesions.

In lambs of group 3 (RSV followed by P. haemolytica in 3 days), 3 lambs killed 4 days post inoculation of the bacteria (PIB) had discrete areas of consolidation and abscesses deep in the lung parenchyma. In the left lung of one lamb there was an area of consolidation with central necrosis and hemorrhages (Fig. 4) resulting from the coalescence of 4 adjacent lesions. This lesion involved more than one-third of the caudal lobe. It was rectangular with extreme diameters of 3.5 cm and 4.5 cm.

The lungs of two lambs killed 2 days PIB had consolidated areas. In one of the lambs, these areas were hemorrhagic and had central necrosis (Fig. 5). There were pulmonary hemorrhages and areas of emphysema or atelectasis in lambs of this group and one lamb also had focal fibrinous pleuritis. There were subendocardial effusions and linear hemorrhages in the left ventricle of one lamb and slight hydropericardium in two others.

In lambs of group 4 (RSV followed by P. haemolytica in 5 days), the lungs had focal areas of consolidation that were either small multifocal pneumonic lesions with well-delineated borders, raised surfaces, firm texture and dark red color, to larger consolidations with hemorrhagic and necrotic centers and peripheral hemorrhage. The first type exuded
fluid from the cut surface on pressure, varied in diameter from 0.4 to 1.5 cm, and was especially evident in the lungs of lambs killed 2 days PIB. These areas were evident in the cranial and caudal lobes. The second type lesion consisted of extensive consolidated areas and was seen in lambs killed 4 days PIB. The second type lesion invariably involved all lung lobes but here also the cranial and caudal lobes were most severely affected. These consolidations varied in size from those involving approximately one-third of the caudal lobe (Fig. 6) and those involving most of the middle lobe to those smaller patches most often seen at the mid-ventral aspect of the cranial and caudal lobes. In 2 lambs, small (0.4 to 2.0 cm) rounded, firm, nonencapsulated abscesses contained a thick semifluid yellow-green exudate. Some of these abscesses protruded above the pleural surface and some were deep in the lung parenchyma. The lungs of all 6 lambs had punctate to 0.2 cm hemorrhages scattered throughout the lung parenchyma, especially in the cranial and caudal lobes. In one lamb they were so numerous as to impart a diffuse hemorrhagic appearance to the lung. There were also foci of emphysema and atelectasis.

Fibrinous deposits on the overlying pleura caused adhesion to the parietal pleura, pericardial sac or between lung lobes themselves. Removal of the fibrin left a raw reddish surface. A similar type of fibrinous pleuritis was seen focally on the parietal pleura. The pleural cavity contained variable amounts of straw-colored fluid that contained some fibrin strands (Fig. 7). There was 2-4 ml of turbid fluid with a few fibrin strands in the pericardial sac of 3 lambs. The pericardial
sac of one lamb, although white and glistening on its parietal part was thickened (up to 0.5 cm) due to a fibrinous deposit on its pericardial mediastinal surface. The mediastinal lymph nodes of all lambs were slightly to moderately enlarged, edematous and congested. One lamb had focal hemorrhages in the cortical portion of the mediastinal lymph node. Tracheas of 4 lambs were congested and contained white froth. In one lamb, the most prominent lesions besides pneumonia were suffusions and petechial hemorrhages in the subpleural, subepicardial, and subendocardial tissues (Fig. 8) and linear hemorrhages in the renal cortices. There was also splenic congestion.

Microscopic lesions In the 4 lambs in group 1 (P. haemolytica alone), the lungs of one were histologically normal, one had only a few focal areas of pneumonitis and two had multifocal areas of acute bronchopneumonia with central coagulative necrosis and fibrinous pleuritis. In the latter 2 lambs the lesions were concentrically zonal. Centrally, the alveoli were defined only by congested capillaries and contained fibrin, proteinaceous material, bacterial colonies, erythrocytes, few neutrophils (PMNs) and macrophages. The central area was surrounded by a zone of darkly basophilic macrophages that were mostly spindle-shaped and oriented parallel to each other. These macrophages formed whorls or bundles that gave the appearance of streaming out of the alveoli. More peripherally the exudate consisted of PMNs and macrophages and became less cellular further peripherally. Outside the cellular zone, there was an abrupt transition to normal lung tissue with no fibrous tissue encapsulation. There were alveoli lined by hyperplastic pneumocytes and
purulent bronchiolitis. The above described lesions encroached upon interlobular septa, and occasionally progressed to frank abscessation. Both the pleura and interlobular septa were thickened by deeply eosinophilic fibrinous deposits and neutrophil infiltrates and contained thrombosed blood vessels and lymphatics.

Severity of lung lesions caused by *P. haemolytica* in the 4 selected sites was graded as follows:

0- Normal

1- Focal atelectasis, emphysema and congestion.

2- Above lesion with increased cellularity in the interstitium or alveolar spaces.

3- Focal consolidation with or without bronchiolitis and peribronchiolar or perivascular lymphocytic cuffing.

4- Acute multifocal fibrinous bronchopneumonia with central necrosis and pleuritis.

According to these criteria, the dorsal portion of the cranial lobe had the most severe lesions and the dorsal portion of the caudal lobe had the least severe lesions. Intermediate in severity were lesions in the ventral portion of the cranial lobe and the ventral portion of the caudal lobe (Appendix, table A-1).

In lambs of group 2 (RSV alone), the lungs had multifocal interstitial pneumonitis (Fig. 9) with the cellular infiltrates being PMNs and macrophages. There was mild to moderate bronchiolitis (Fig. 10) as well as occasional examples of bronchitis. The mucosa of the airways was hyperplastic and there was peribronchiolitis characterized by accumulations of macrophages and PMNs. There were alveolar macrophages free in the alveolar lumen, congested alveolar capillaries, occasional necrotic alveolar lining cells (Fig. 11) and focal consolidation, mostly adjacent
to affected bronchioles. These focal consolidations consisted predomi-
nantly of macrophages along with lymphocytes and a few PMNs.

The adjacent lung tissue was either normal or had slightly increased
cellularity due to the presence of excessive numbers of alveolar macro-
phages and to hyperplasia and hypertrophy of pneumocytes. There were
focal areas of hemorrhage, and atelectatic and emphysematous areas.
Occasionally, multinucleated giant cells were seen in the alveolar
spaces (Fig. 12) but no inclusion bodies were detected with H&E or
special stains.

The severity of lung lesions caused by RSV in the 4 selected sites
was graded as follows:

0- Normal.
1- Interstitial pneumonitis.
2- Above lesion with bronchiolitis.
3- Above with hemorrhages or peribronchiolar lymphoid cuffs,
or both.

The ventral portions of the cranial lobe and of the caudal lobe had
slightly more severe lesions than the dorsal portions of the same lobes
(Appendix, table A-2).

In both the trachea and especially nasal turbinates, there was focal
erosion to ulceration of the surface epithelium. Polymorphonuclear cells
were seen focally infiltrating the epithelium and lamina propria of the
trachea and turbinates and extending to the congested submucosa of the
latter.

All lambs in groups 3 and 4 (RSV followed by P. haemolytica in 3 or
5 days) had (1) multifocal areas of interstitial pneumonitis, and (2)
pulmonary consolidation. The interstitial pneumonitis consisted mainly
of macrophages, lymphocytes and few PMNs. There were also focal areas of alveolar necrosis, hyperplasia and hypertrophy of alveolar lining cells, and occasional multinucleated giant cells. In the peribronchiolar and perivascular connective tissue there was slight to marked lymphoid hyperplasia.

The pulmonary consolidations were small and usually peribronchiolar or were larger and more extensively involved pulmonary parenchyma without particular orientation to bronchioles. In both, the cellular components were macrophages with few lymphocytes and PMNs scattered throughout. The consolidated areas had alveolar necrosis and purulent bronchiolitis with hyperplasia of the bronchiolar epithelium. Adjacent to the consolidated areas there were foci of atelectasis as well as foci of emphysema. All the above lesions were seen in lambs killed 2 days PIB of group 4 and in 3 lambs of group 3 (2 of them killed 4 days PIB).

A third type of lesion (Figs. 13 and 14) was seen in all 3 lambs killed 4 days PIB from group 4 and in 2 lambs killed 2 and 4 days PIB from group 3. This lesion was similar to the concentrically zonal lesion observed in lambs infected with P. haemolytica alone but differed from it by having a thicker inflammatory cell (macrophages and PMNs) zone outside the streaming pleomorphic cell zone. Other lesions observed in the above 5 lambs were: frequent and pronounced lymphocytic cuffs about vessels and bronchioles, congestion and thrombosis of both blood and lymphatic vessels, bronchiolar epithelial hyperplasia, hyperplasia and hypertrophy of alveolar lining cells, and syncytia cell formations.
(Fig. 15). The latter were not seen in lambs infected with *P. haemolytica* alone and were more frequent in lambs killed 4 days PIB than 2 days PIB.

Vasculitis of small-sized pulmonary arterioles was seen in all lambs from group 4 and 3 lambs of group 3. In lambs of both groups, there was a severe purulent bronchiolitis.

Severity of lung lesions caused by the combined infection in the selected sites was graded as follows:

0 - Normal.
1 - Focal atelectasis with or without cellular infiltration, emphysema or hemorrhage.
2 - Interstitial pneumonitis with or without lymphocytic cuffs, hemorrhage and bronchiolitis.
3 - Multifocal bronchopneumonia, peribronchiolitis and lymphocytic cuffs.
4 - Extensive bronchopneumonia, with or without suppuration and perivascular and peribronchiolar lymphocytic cuffs.
5 - Multifocal bronchopneumonia with central necrosis and fibrinous pleuritis (*Pasteurella*-type pneumonia).

The lesions were generally more severe in the dorsal portions of the cranial lobe of lambs of both groups. The other three regions were similarly but somewhat less severely affected (Appendix, table A-3).

In both the tracheas and nasal turbinates of 10 of 11 lambs a similar kind of lesion to that described in the lambs infected with RSV alone was seen in lambs with dual infection. Mild cryptitis was seen occasionally in the submucosa of the nasal turbinates. There was slight to moderate reactive hyperplasia of the bronchial lymph nodes of lambs of the two groups.
Discussion

In lambs infected with *P. haemolytica* alone, the salient lesions were fibrinous pleuritis and exudative pneumonia with extensive consolidation, hemorrhages, focal necrosis, and streaming pleomorphic inflammatory cells in air spaces. These lesions have also been reported in natural cases of pneumonic pasteurellosis or enzootic pneumonia of sheep, and supports previous reports that *P. haemolytica* alone can induce severe pulmonary lesions in sheep.

Lesions caused by infection with RSV alone consisted of multifocal areas of consolidation and hemorrhage, focal interstitial pneumonitis and slight to moderate bronchiolitis. These findings are in agreement with previous reports of experimental infections with bovine RSV in lambs, in calves, and in natural infection of children with RSV.

Pneumonic lesions were seen in all lambs given both RSV and *P. haemolytica*. The lesions were more severe than those produced by either of the agents given alone but were qualitatively similar to those seen in lambs infected with *P. haemolytica* alone. The lesions conformed in all morphological aspects to those described in natural cases of acute pneumonic pasteurellosis in sheep and in cattle. They were not different from the experimentally produced disease in lambs and calves. The lesions also conformed in all histopathologic features to those described in natural cases of ovine pneumonic pasteurellosis and were compatible with those reported in experi-
mental production of pneumonic pasteurellosis in adult sheep and lambs and in calves using \textit{P. haemolytica} alone. The lesions were also similar to those of lambs experimentally infected with a combination of PI-3 virus and \textit{P. haemolytica} described by Rushton et al. However, these workers described an additional connective tissue zone to the concentrically zonal lesions we described in this experiment. This could be attributed to the longer period of time elapsed between infection and euthanasia in their experiments (up to 16 days post inoculation of PI-3 and up to 9 days PIB).

In addition to the \textit{Pasteurella}-type lesion, some of the lungs of lambs of the combination groups had some features characteristic of infection with RSV alone. These were hyperplasia of bronchiolar epithelium and of alveolar lining cells, peribronchiolar and perivascular lymphocytic cuffs and syncytia formation. The viral-induced lesions underlay the more severe bacterial-induced lesions.

The necrotic changes observed in lambs with dual infection are, in our opinion, the result of vasculitis leading to ischemia. We recognize, however, that direct bacterial action may have been responsible for, or contributed to the observed necrosis. Acute vasculitis and thrombosis have been reported in lambs infected with \textit{P. haemolytica}, and were seen in most lambs given both agents in the present study but no vasculitis was seen in lambs given either agent alone.

Pulmonary lesions in the lambs of the combination groups were slightly more severe histologically in the dorsal portions of the cranial lobe than in the other three areas examined. This was, indeed,
the site from which the most bacteria were recovered from the lungs of lambs given both agents, and was also the site of greatest lesion severity in the lambs given \textit{P. haemolytica} alone.

Regarding the role of RSV in the production of the lesion observed in lambs with dual infection, we propose that the virus initiated a lesion of sufficient severity to compromise lung vitality, permitting \textit{P. haemolytica} to become established and to produce a more severe pneumonic lesion than could be elicited by that organism alone.
Fig. 1. An area of consolidation in the center of the cranial lobe of the left lung from a lamb inoculated with *P. haemolytica* and killed 4 days postinoculation. Note the hemorrhagic and necrotic center and the dark peripheral ring of congestion and hemorrhage.

Fig. 2. Roughly spherical, 3.5 cm diameter area of consolidation in the ventral part of the cranial lobe of the left lung from a lamb inoculated with *P. haemolytica* and killed 2 days postinoculation.
Fig. 3. Multifocal areas of consolidation involving most of the proximal third of the caudal lobe of the right lung from a lamb inoculated with RSV and killed 5 days postinoculation. The areas are slightly raised and there are numerous petechial hemorrhages.

Fig. 4. Extensive pneumonic lesion made up of 4 adjacent consolidated areas with necrotic and hemorrhagic centers. More than one-third of the caudal lobe of the left lung is involved. The lung is from a lamb inoculated with RSV followed in 3 days by inoculation with P. haemolytica and killed 4 days later.
Fig. 5. Focally extensive areas of consolidation with hemorrhages in the antero-ventral portion of the caudal lobe of the right lung from a lamb inoculated with RSV followed in 3 days by inoculation with *P. haemolytica* and killed 2 days later.

Fig. 6. Multifocal areas of consolidation with hemorrhages in the caudal lobe of the right lung from a lamb inoculated with RSV followed in 5 days by inoculation with *P. haemolytica* and killed 4 days later. Note the lobular distribution.
Fig. 7. Hydrothorax in a lamb inoculated with RSV followed in 5 days by inoculation with P. haemolytica and killed 2 days later. Note the fluid level dorsal to the lung.

Fig. 8. Subendocardial hemorrhage in a lamb inoculated with RSV followed in 5 days by inoculation with P. haemolytica and killed 2 days later.
Fig. 9. Interstitial pneumonitis in a lamb inoculated with RSV and killed 5 days later. The thickening of alveolar septa is due to accumulations of mononuclear cells. H and E, 180X.
Fig. 10. Bronchiolitis in a lamb inoculated with RSV and killed 5 days later. Note sloughed and disrupted bronchiolar epithelium and peribronchiolitis. H and E, 300X.
Fig. 11. Lung from a lamb inoculated with RSV and killed 7 days later. There is interstitial thickening and pulmonary consolidation. Note the necrotic cellular debris in alveolar lumens, necrosis of alveolar walls, and the adjacent emphysema. H and E, 300X.
Fig. 12. Multinucleated giant cells in the pulmonary alveolar lumen of a lamb inoculated with RSV and killed 5 days later. H and E, 1200X.
Fig. 13. Lung from a lamb inoculated with RSV followed in 5 days by inoculation with *P. haemolytica* and killed 4 days later. There is an acute, severe exudative pneumatic lesion with central necrosis. Centrally the alveoli are defined only by distended capillaries. H and E, 120X.
Fig. 14. Lung from a lamb inoculated with RSV followed in 5 days by inoculation with *P. haemolytica* and killed 4 days later. The lesion consists of necrotic parenchyma, proteinaceous exudate within alveoli, congested alveolar capillaries and streaming arrays of rounded to elongated cells. H and E, 300X.
Fig. 15. Lung from a lamb inoculated with RSV followed in 3 days by inoculation with *P. haemolytica* and killed 2 days later. Multinucleated giant cell in an area of consolidation. H and E, 750X.
References


PART III. EXPERIMENTAL INFECTION OF LAMBS WITH RESPIRATORY Syncytial VIRUS AND Pasteurella Haemolytica -- IMMUNOFluorescent AND ELECTRON MICROSCOPIC STUDIES

This manuscript will be submitted to the American Journal of Veterinary Research.
Summary

Colostrum deprived lambs were inoculated with respiratory syncytial virus (RSV), Pasteurella haemolytica or both agents. There was a 3 or 5 day interval between viral and bacterial inoculation in those lambs receiving dual inoculation. Samples of nasal turbinates, trachea, mediastinal lymph node, lungs, and spleen were examined by immunofluorescence to localize viral and bacterial antigens. Consolidated and apparently normal areas of lungs were examined ultrastructurally.

In lambs inoculated with RSV alone or with both agents, specific immunofluorescence of viral antigen was limited to the respiratory tract. Viral antigen was detected primarily in the bronchial and bronchiolar epithelium and in the alveolar wall. Lesser amounts of viral antigen were detected in the surface epithelium of the nasal turbinates and trachea. No bacterial antigens were detected in tissues of lambs inoculated with P. haemolytica alone or in combination with RSV.

Ultrastructurally, the lambs infected with P. haemolytica alone or with both agents had increased numbers of type II pneumocytes, necrotic epithelial cells, neutrophils, macrophages and cellular debris. Although bacteria were only infrequently encountered, they were found intracellularly within neutrophils and macrophages and occasionally free within the alveolar lumen. In lambs infected with RSV alone or with both agents, there were multinucleated giant cells and virus particles budding from bronchial and bronchiolar epithelial cytoplasmic membranes. Budding occurred from both ciliated and nonciliated epithelial cells and
isolated epithelial cells were necrotic. Viral nucleoprotein was present in some alveoli either free or within vacuoles of phagocytic cells. Necrotic debris and phagocytic cells were more prominent in the alveoli of lambs with combined viral-bacterial infection than in those of lambs infected with either agent alone.
Introduction

*Pasteurella haemolytica* is commonly associated with respiratory disease in sheep, but the experimental production of pneumonia in sheep with this organism alone has proven difficult. 1-3 Viruses are among the agents claimed to predispose sheep lung to *Pasteurella haemolytica* infection. Parainfluenza type 3 (PI-3) virus, for example, is reported to increase the severity of lesions caused by *P. haemolytica* in conventional lambs 4 and to accurately reproduce the lesions of acute pneumonic pasteurellosis when given in combination with *P. haemolytica* to specific pathogen-free lambs. 5,6

Respiratory syncytial virus (RSV) was first described under the name of chimpanzee coryza agent by Morris et al. 7 in 1956. Later, it was isolated from human infants with severe respiratory infection, 8 and its importance in the etiology of human respiratory illness was established. 9-11

A virus designated "bovine RSV" has been isolated from the respiratory tract of cattle in Switzerland, 12 Japan, 13 Belgium, 14 England, 15 Denmark, 16 and the USA. 17-19 Bovine RSV was shown to be an important cause of respiratory disease in cattle. 17,18,20-22 Both complement-fixing and neutralizing antibodies to bovine RSV were reported in sheep sera. 21,23

Experimental infection of animals with human RSV resulted in different patterns of viral isolation and viral antigen localization which sometimes were dependent on the age of the animal at the time of
inoculation and on the particular tissues examined. In ferrets, for example, immunofluorescence revealed abundant viral antigens in the nasal epithelium and in scattered pulmonary alveolar cells with the lower airways being spared. This was in contrast to the infection of cotton rats where viral antigens were located primarily in the bronchiolar epithelium and not the pulmonary alveolar cells. The trachea was free from immunofluorescence. In the cebus monkey, however, viral antigens were detectable in alveolar cells throughout the lung and in the bronchiolar epithelium and to a lesser extent in the trachea epithelium.

In children with RSV infection of the lower respiratory tract, immunofluorescence was detectable in bronchial and bronchiolar epithelium and alveolar lining cells as well. In cattle with pulmonary lesions, bovine RSV antigens were located in epithelial cells of the bronchi and bronchioles and in the alveolar lumen.

The cytopathology in the respiratory epithelium infected with human RSV has not been adequately described. However, it was concluded from in vitro studies in cell cultures that severe cell swelling was accompanied by cytoplasmic accumulation of nucleocapsid material and by budding of virus particles from the plasma membrane. Cutlip and Lehmkuhl experimentally infected colostrum-deprived lambs with bovine RSV. They reported that ultrastructural changes consisted of multinucleated giant cells, increased numbers of granular pneumocytes and formation of granular intracytoplasmic inclusion bodies in epithelial
cells. Virions were free in alveoli and in bronchioles and were budding from cytoplasmic membranes of the latter.

This project was undertaken to study by immunofluorescence and electron microscopy the distribution and cytopathology of RSV and P. haemolytica within the respiratory tract of experimentally infected sheep.

Materials and Methods

The experimental design has been previously outlined in detail. Briefly, 22 colostrum-deprived, 4-week-old lambs were divided into 5 groups and given RSV or P. haemolytica alone or in combination or sterile inoculum by the transtracheal route. Groups 1 and 2 with 4 lambs each were given P. haemolytica or bovine RSV respectively. Groups 3 and 4 with 6 lambs each were given both agents with 3 and 5 days delay between viral and bacterial inoculation respectively. The bacterial inoculum was 5 ml of culture fluid with a viable cell count of $3 \times 10^7$ colony-forming units/ml and the viral inoculum was 20 ml of $2.9 \times 10^4$ plaque-forming units/ml. Group 5, a control group with 2 lambs, was given sterile tissue culture fluid and sterile bacterial culture fluid.

**Immunofluorescence** Antiserums to RSV and P. haemolytica were prepared in sheep and rabbits respectively. Immunoglobulin (Ig) for conjugation with fluorescein isothiocyanate (FITC) was purified by 3-time precipitation with 70% ammonium sulfate followed by dialysis against frequent changes of 0.85% NaCl solution (pH 8.0) until sulfate was no longer detected in the dialysate. The purified Ig was mixed with FITC
at the ratio of 25 μg of FITC/mg of the Ig. The unbound dye was separated from free Ig and FITC-labelled Ig on a gel column.\(^a\)

At necropsy, samples from the following organs were collected: nasal turbinate, trachea, lung, mediastinal lymph node and spleen. The tissues were embedded in 2.5% methyl cellulose, frozen and 6 μm sections were cut on a cryostat microtome. Sections were fixed in acetone and were stained indirectly or directly with FITC-labelled immunoglobulin to bovine RSV and to \(P. \text{haemolytica}\) respectively according to the methods described by Coons.\(^32\)

In the indirect fluorescent staining technique, rabbit anti-sheep antiserum,\(^b\) sheep anti-RSV antiserum and fluorescein-conjugated rabbit anti-sheep antiserum\(^b\) were used. In the direct method, a fluorescein-conjugated rabbit anti-\(P. \text{haemolytica}\) antiserum was used.

Specificity of the reaction was evaluated by running a similar control section from noninfected lambs and by blocking positive tissues with homologous unlabeled antiserum. The fluorescein-conjugated rabbit anti-\(P. \text{haemolytica}\) antiserum activity was tested by staining a smear of the bacteria used in the inoculum. Sections were incubated with specific conjugate or antiserum as required for staining or blocking, in a humid chamber at 37°C for 30 minutes and rinsed for 5 minutes in each of three changes of Dulbecco's phosphate buffered saline.

\(^a\)Sephadex G-25, Pharmacia Fine Chemicals, Inc., Piscataway, NJ
\(^b\)Miles Laboratory Inc. Research Division, Elkhart, IN
Electron Microscopy  Tissues for electron microscopy were taken from consolidated and apparently normal lungs. Abscesses and necrotic foci were not examined. The tissues were fixed in 2.5% glutaraldehyde in cacodylate buffer (pH 7.2). The tissues were post-fixed in 1% osmium tetroxide, dehydrated in graded ethanol, and embedded in epoxy resin. Ultrathin sections were cut on an ultramicrotome, stained with lead citrate and uranyl acetate and examined in a Philips 200 electron microscope.

Results

Immunofluorescence  Abundant viral antigen was detected in the lungs of lambs infected with RSV (Fig. 1) and with both RSV and P. haemolytica (Fig. 2). In both cases, viral antigen was seen as solid sheets or as multiple foci of fluorescence in epithelium of the bronchi and bronchioles and, to a lesser extent, within the cytoplasm of cells in the alveolar and bronchiolar lumens. No viral antigen was seen in alveolar epithelium. More frequent and more intense specific fluorescence was seen in lung tissues from lambs killed earliest in the course of the experiment.

Viral antigen was detected in the surface epithelial cells of the nasal turbinates and trachea but fewer cells fluoresced in these sites than in the lower respiratory tract.

No viral antigen was detected in spleen or mediastinal lymph nodes from lambs infected with RSV alone or in combination with P. haemolytica.

^Epon 812, Shell Chemical Co., Kansas City, MO
Specific RSV fluorescence was not detected in tissues from lambs infected with *P. haemolytica* alone or in tissues from the controls.

There was no specific *P. haemolytica* fluorescence in tissues from lambs infected with *P. haemolytica* alone or in combination with RSV or in tissues from the controls. Smears of the cultures of *P. haemolytica* A-1 used as the experimental inoculum demonstrated positive specific immunofluorescence.

**Electron microscopy** Many ultrastructural changes in the lungs of lambs infected with *P. haemolytica* alone or with RSV and *P. haemolytica* were similar. In these, there were increased numbers of type II pneumocytes and in sections taken from consolidated areas the alveolar and bronchiolar lumens were filled with necrotic cellular debris, neutrophils, macrophages, and erythrocytes (Fig. 3). Phagocytic cells contained a few degenerate intracellular bacteria (Fig. 3) and bacteria occasionally were seen free in alveolar lumens. Less frequently, extracellular bacteria were observed in interalveolar septa (Fig. 4).

In lambs inoculated with RSV there were a few multinucleated giant cells. Cellular changes in lambs given RSV alone were not as extensive as those in lambs given both agents.

In lambs infected with RSV alone or with both agents, virus was seen budding from the plasma membrane of ciliated and nonciliated epithelial cells (Fig. 5 and 6) of the bronchi and bronchioles but not from membranes of the pneumocytes. Numerous complete viral particles were seen in the lumens of these airways (Fig. 5) and less frequently in
the alveolar lumens. The complete virions were pleomorphic; most were
round or filamentous. The wall consisted of a trilaminar membrane
covered with surface spikes and the core contained a set of fine fibrils
of nucleoprotein arranged in a circular pattern. Complete virions were
120 to 155 nm (average 139 nm) in diameter and the surface spikes were
10 to 15 nm (average 12 nm) long. Some filamentous forms appeared to
represent altered microvilli with central fibrils of nucleoprotein core
and surface spikes (Fig. 7). On these altered microvilli, zones of
membrane with spikes alternated with zones devoid of spikes. Viral buds
varied in shape and size from a localized thickening of cytoplasmic
membrane overlying dot-like viral nucleoprotein to complete enveloped
virions attached to the cell by a slender stalk (Fig. 5, 6, and 7).

Individual epithelial cells in the bronchi and bronchioles were
necrotic. Some of the necrotic cells had small, electron dense, pre­
sumably degenerate, viral particles associated with their surfaces (Fig.
8). Numerous aggregates of viral nucleoprotein were seen in bronchioles
and alveoli mixed with cellular debris and within membrane-bound vacuoles
in macrophages and neutrophils (Fig. 3). Nonphagocytized viral in­
clusions were not identified with certainty within cells.

Incidental to the lesion, a ciliated bronchiolar epithelial cell
was observed which contained membrane-bound fibrogranular inclusions
(Fig. 9). There were no ultrastructural lesions in nonconsolidated
areas of lungs or in the lungs of control lambs.
Discussion

The results of immunofluorescence studies reported in this experiment are in agreement with previous work on localization of bovine RSV antigen in cattle and of human RSV in cotton rats and cebus monkeys. Specific fluorescence in lung tissues from lambs infected with RSV was more abundant when compared to that seen in nasal epithelium or trachea. This finding is compatible with the observations that fewer viral isolations were obtained from the nasal turbinates than from the lungs and that only mild focal rhinitis and tracheitis were reported. We propose that the specific intense fluorescence seen intracellularly correlates to the viral nucleoprotein seen ultrastructurally in phagocytic cells. The finding of more frequent and more intense fluorescence in lung tissues from lambs killed earlier in the experiment may reflect a time-related maximal viral replication and hence maximal intracellular antigen accumulation. Evidence of that possibility was present in the results of viral isolations reported earlier.

Failure to detect bacterial antigen by immunofluorescence probably reflects, in part, the rapidity with which P. haemolytica is removed from the lungs. Supporting that contention is the unusually low numbers of bacteria seen in specially stained histological sections and in sections examined ultrastructurally in which most of the bacteria were engulfed and degenerate and probably of a sufficiently altered antigen structure as not to be detectable by immunofluorescence.
Ultrastructurally, the lesions of uncomplicated RSV infection were compatible with previous reports of experimental infection of sheep with bovine RSV. The patterns of viral growth and the viral morphology reported in this experiment were similar to those reported for human and bovine RSVs in cell cultures and for bovine RSV in sheep lung.

From our ultrastructural observations we propose that the primary targets of infection of lambs by bovine RSV are the epithelial cells of bronchi and bronchioles. Infection and destruction of these cells predispose the lung to subsequent infection by P. haemolytica.

The bronchiolar ciliated epithelial cell with fibrogranular inclusions is reported for the first time in sheep lung. This type of cell has previously been reported only in the bonnet monkey (Macaca radiata). The inclusions were believed to be a secretory product.
Fig. 1. Fluorescence (RSV) in bronchiolar epithelium of a lamb inoculated with RSV and killed 5 days postinoculation. Stained by indirect immunofluorescent technique for RSV. 930X.

Fig. 2. Fluorescence (RSV) in the consolidated lung tissue of a lamb inoculated with RSV and *P. haemolytica*. Note the fluorescence within macrophages (short thin arrow), free in alveoli (long thin arrow) and within epithelium of a bronchiole (heavy short arrow). Stained by indirect immunofluorescent technique for RSV, 480X. The cells containing immunofluorescent material were identified by restaining with H and E.
Fig. 3. Electron micrograph from an area of consolidation of the lung of a lamb inoculated with RSV and P. haemolytica. The alveolar space is filled with neutrophils (N), a macrophage (M), and necrotic debris (ND). Note the viral nucleoprotein (NP) within a neutrophil and free within the lumen and an intracellular degenerate bacterial cell (DB). A capillary in the alveolar wall is to the lower left. 6,200X.

Fig. 4. Electron micrograph of 2 bacterial cells near the end stage of binary fission. They are in an interalveolar septum of the lung of a lamb inoculated with P. haemolytica. The alveolar space is in the top right corner and it contains a neutrophil. 24,000X.
Fig. 5. Bronchiolar ciliated cell with virus budding from the plasma membrane and with complete virions in the bronchiolar lumen. Virions are distinguished by the spikes, trilaminar membrane and a core of electron-dense nucleoprotein fibrils. The fibrils are also seen within the cell cytoplasm just below the viral buds (arrows). 92,000X.

Fig. 6. RSV buds (arrows) on a bronchiolar goblet cell membrane from a lamb inoculated with RSV and P. haemolytica. 41,000X.
Fig. 7. Bronchiolar ciliated epithelium from the lung of a lamb inoculated with RSV and P. haemolytica. Microvilli contain internal fibrils and surface spikes believed to be of viral origin (arrows). 41,000X.

Fig. 8. An isolated necrotic epithelial cell in a bronchiole from a lamb inoculated with both RSV and P. haemolytica. Note the dense viral particles at the luminal surface of the cell (arrows). 11,000X.
Fig. 9. A ciliated bronchiolar epithelial cell from a lamb inoculated with both RSV and *P. haemolytica*. Note multiple cytoplasmic fibrogranular inclusions (arrows). 18,000X. Inset: Enlarged inclusion. 121,000X.
References


GENERAL DISCUSSION AND CONCLUSIONS

The results of experimental infection of colostrum-deprived lambs with a combination of RSV and P. haemolytica reported in this study showed that a more severe clinical disease was produced in lambs given both agents than was produced in lambs given either agent alone. The clinical signs of illness caused by combined infection were pyrexia, hyperpnea, dyspnea, nasal discharge, and coughing. A natural outbreak of respiratory disease in cattle was recently reported; the etiology was proposed as combined RSV-P. haemolytica infection. There is no reference to the significance of the combined effect of RSV and P. haemolytica on the ovine lung. This is true even though P. haemolytica can be isolated and a serologic response to RSV can be detected. The respiratory disease in lambs with dual infection reported in this study is similar to that reported in lambs infected with a combination of PI-3 and P. haemolytica. The study showed that the most severe signs result when exposure to P. haemolytica occurs five days following exposure to RSV.

Only mild respiratory disease was observed in some lambs inoculated with RSV alone and indeed was seen in only one lamb inoculated with P. haemolytica. These signs were not different from those previously reported in lambs infected with the two agents.

The results of bacterial isolation indicated that RSV may have enhanced the spread of P. haemolytica within the lung since the latter was isolated three and four times from the dorsal and ventral portions
respectively of the caudal lobes of lungs of lambs inoculated with both agents. This was in contrast to no bacterial isolations from these sites in lambs inoculated only with the bacteria.

The results of viral isolation supported previous reports that RSV was difficult to isolate \(^9,20,22\) and that the pulmonary tissue was the primary site for its replication.\(^10\)

The pathological findings of this study showed that the combined infection produced more severe lesions than those produced by either agent given alone (Appendix, table A-1 through table A-3). The gross lesions caused by the combined RSV-P. haemolytica infection were consolidation, fibrinous pleuritis, hydrothorax, and hemorrhages of the serous surfaces. The microscopic lesions were characterized by exudative focal necrosis; alveoli packed with elongated pleomorphic macrophages, proteinaceous exudate with fibrin, erythrocytes, Gram-negative coccobacilli, and small focal areas of PMN leukocytes but not in the form of extensive suppuration. The above gross and microscopic lesions were also reported in ovine and bovine pneumonic pasteurellosis.\(^4,5,86,87\)

The relatively high incidence of antibodies to RSV in sheep \(^12,13\) coupled with RSV's ability to produce a mild pneumonitis\(^11\) suggest that the virus could be significant in predisposing sheep to infection with P. haemolytica which is normally present in the upper respiratory tract of sheep. The combined infection could lead to a severe Pasteurella-type pneumonia.
This study showed that *P. haemolytica* alone is capable of producing severe pneumonic lesions. This is in agreement with previous work on lambs.\(^72\)\(^{-74}\)

Lesions caused by RSV in lambs in this study are similar to those previously reported in week-old\(^10\) and feeder lambs,\(^39\) in calves,\(^36\) and in children in natural outbreaks of human RSV infection.\(^40\)

The present electron microscopic and immunofluorescence studies suggest that pulmonary tissue is permissive of RSV infection and replication; indeed, the ciliated and nonciliated epithelium of large and terminal airways were infected with virus. Among these cells, isolated cells were necrotic, had virus budding from their plasma membranes or along their microvilli, and contained numerous granular inclusion bodies. Bronchiolar and alveolar lumens contained numerous virus particles. It is thought that the source of virus present in alveoli was the productively infected bronchioles. Evidence supporting that concept was the absence of budding virus from the alveolar epithelium. The predominant location of the viral antigens was the bronchi and bronchioles. This is in agreement with the results of previous studies on the cotton rat, cebus monkey,\(^8\) and cattle.\(^32\) The patterns of virus growth and the viral morphology reported in this experiment are similar to those reported by other workers.\(^11\) From the ultrastructural findings, it is proposed that the primary targets of infection of lambs by bovine RSV are the epithelial cells of bronchi and bronchioles. Infection and destruction of these cells predispose the lung to subsequent infection by *P. haemolytica*. 

Design of this experiment was not adequate to study all aspects of infection with each agent used separately and together. Ideally, to more accurately determine ultrastructural changes in RSV infection, the lungs of a series of lambs should be studied earlier in the course of infection than was done in the present study.

The fact that infection of lambs with RSV was symptomatic and that the lesions were comparable to those seen in children infected with human RSV, suggests that the lamb is an adequate model for studying the important lower respiratory disease of infants and children that is caused by human RSV. It would be important to determine whether sheep react to reinfection with bovine RSV in the same way (aberrant immune-mediated reaction) that humans do to reinfection with the human virus. One approach to answering the above question would be immunization of lambs and subsequent virus challenge.
ADDITIONAL REFERENCES CITED


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A special note of thanks and gratitude is extended to my wife, Betool, for her encouragement and endurance during the period of my graduate study.
### Table A-1. Severity of Lung Lesions at 4 Sites from Lambs of Group 1 (P. haemolytica alone)*

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<th>Caudal lobe</th>
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<td>Dorsal</td>
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<tr>
<td>Average</td>
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</tbody>
</table>

*Criteria for grade evaluation:
0- Normal
1- Focal atelectasis, emphysema and congestion
2- Above lesion with increased cellularity in the interstitium or alveolar spaces
3- Focal consolidation with or without bronchiolitis and peribronchial or perivascular lymphocytic cuffing
4- Acute multifocal fibrinous bronchopneumonia with central necrosis and pleuritis
Table A-2. Severity of Lung Lesions at 4 Sites from Lambs of Group 2 (RSV alone)*

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<th>Caudal lobe</th>
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</tbody>
</table>

*Criteria for grade evaluation:

0- Normal
1- Interstitial pneumonitis
2- Above lesions with bronchiolitis
3- Above with hemorrhages or peribronchiolar lymphoid cuffs, or both
Table A-3. Severity of Lung Lesions at 4 Sites from Lambs of Groups 3 and 4 (RSV followed in 3 or 5 days, respectively, by P. haemolytica)*

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<th>Caudal Lobe</th>
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<td>Average</td>
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<td>2.6</td>
<td>2.6</td>
<td>2.6</td>
<td></td>
</tr>
</tbody>
</table>

*Criteria for grade evaluation:

0- Normal
1- Focal atelectasis with or without cellular infiltration, emphysema or hemorrhage
2- Interstitial pneumonitis with or without lymphocytic cuffs, hemorrhage and bronchiolitis
3- Multifocal bronchopneumonia, peribronchiolitis and lymphocytic cuffs
4- Extensive bronchopneumonia, with or without suppuration and perivascular and peribronchiolar lymphocytic cuffs
5- Multifocal bronchopneumonia with central necrosis and fibrinous pleuritis (Pasteurella-type pneumonia)