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Title: VIRUSES ASSOCIATED WITH RESPIRATORY DISEASE: A STUDY OF SHEEP IN AN OREGON AND COLORADO FEEDLOT

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Abstract approved:

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A study was undertaken to determine the prevalence of virus infection in lambs undergoing respiratory tract disease (RTD). The investigation was divided into three study groups and consisted of lambs which were 4 to 5 months of age. In the first group (N=200), paired serum samples were taken from lambs (on entry into the feedlot and 21 days later). In addition, lambs in the group which died of RTD were necropsied and tissue was cultured for the presence of bacteria and examined for viral antigen by fluorescence microscopy, (respiratory syncytial virus, RSV; parainfluenza-3 virus, PI-3; border disease virus, BDV; and ovine adenovirus-6, OVA-6). During the 21 day test period, 47 lambs died of RTD but only 40 were available for study. Antigen to RSV was detected most frequently (37.5% of cases). This was followed by PI-3 (23%), OVA-6 (15%) and BDV (7.5%). More than one virus was detected in 20% of cases. Ten different bacteria were isolated from lung tissue. There were not enough isolations of bacteria to statistically associate a specific type of bacteria with any of the viruses. Paired serum samples from surviving lambs (N=153) were examined for seroconversion to the viruses tested above plus blue tongue virus (BTV). Seroconversion to PI-3 was most commonly observed (47% of cases). This was followed by RSV (35%), OVA-6 (16%), BDV (5%) and BTV (2%).

The second Oregon study involved a seroprevalence from a representative number of lambs (N=50) from five different pens in the feedlot at the same time as the first study (each pen contained 200 lambs). Serum samples were taken as lambs entered the feedlot and a second sample (N=50) was taken from lambs from same group 24 days later. A significant increase (P=.001 to .05) in the percentage of lambs possessing antibodies to the test viruses was detected in all cases. The most dramatic change occured with RSV (36% to 88% prevalence) and OVA-6 (48% to 86% prevalence). Changes in percentage of lambs with antibodies to PI-3 and BDV were less dramatic.

The third study was undertaken in a Colorado feedlot. Serum samples were taken as lambs entered the facility (N=200) and a second (paired) serum sample was taken 21 days later from the same lambs. Nineteen lambs died of RTD but they were not examined for presence of viral antigen. Seroconversion in surviving lambs was highest with PI-3 (51%). This was followed by OVA-6 (19%), RSV (17%) and BTV (1%).

VIRUSES ASSOCIATED WITH RESPIRATORY DISEASE: A STUDY OF SHEEP IN AN OREGON AND COLORADO FEEDLOT.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

VIRUSES ASSOCIATED WITH RESPIRATORY DISEASE: A STUDY OF SHEEP IN AN OREGON AND COLORADO FEEDLOT.

I. INTRODUCTION

Diseases affecting the respiratory system of sheep and goats are relatively common. It is probably accurate to state that respiratory disease contributes more to morbidity and mortality among young small ruminants than does any other infectious disease.

Most authorities agree that the etiology of respiratory disease in ruminants is multifactorial and among those the virus involvement is a major primary cause. The viruses that appear to have the greatest potential to predispose lambs to respiratory diseases includes adenovirus, parainfluenza-3, border disease virus and respiratory syncytial virus. All of these viruses usually cause mild respiratory disease with fever, anorexia, sporadic coughing, pneumonia and listlessness.[107]

Respiratory disease is not limited to USA but is a worldwide problem. The author is a citizen of Pakistan and when he returns to his country he will be incharge of developing biologics for prevention of diseases in sheep and goats. Livestock production is the principal occupation of the inhabitants of Balochistan (PAKISTAN). About 80% of the people depend directly or indirectly on livestock rearing. Sheep and goats are the major livestock species of the province. There are 11.1 million sheep and 7.4 million goats in the province which constitutes 40% and 23% of total country's sheep and goats respectively.

Overall productivity of these animals is low due to poor feeding, management and prevalence of various viral, bacterial and parasitic diseases. These animals

acquire 90% of their feed requirements from grazing on rangeland where the forage availability is very low. This, in turn, predisposes the animals to various infectious diseases.

The economic effects of respiratory diseases in sheep and goats include mortalities as well as indirect estimators of morbidity such as increased time to reach market weight, poor feed conversion, higher rates of culling, poor carcass composition, increased condemnation at slaughter and extra cost and time for medication and veterinary services.[107]

The present investigation was designed to study the viral causes of respiratory diseases in sheep in two feedlots in the United States. Using the information and diagnostic skills in the current study as a model, the author plans to establish diagnostic and research projects in his native country in order to control some of the respiratory problems in sheep and goats.

II. LITERATURE REVIEW

Respiratory tract disease in sheep is a complex syndrome caused by a number of infectious microorganisms plus the interaction of environment, nutritional, and other poorly defined factors.[107] In attempting to determine the role of viruses in this disease, serologic prevalence as well as virus isolation studies are of value. Using these methodologies, individual viruses as well as groups of viruses which naturally infect an animal can be determined. Subsequently, more detailed investigations can then be undertaken to define the significance of each virus which has been shown to infect the specie in question. These more detailed studies may involve serologic response to a virus taken at specific times to determine sequence of infection. This is then correlated with appearance of naturally occurring disease. Additional studies may also involve attempts to reproduce the disease by experimental inoculation of a virus into a group of animals and to determine the pathogenesis of infection.

A comprehensive literature review of all viral agents which may cause respiratory tract disease in the sheep would be an overwhelming task and beyond the scope of this study. Accordingly, a general summary of the importance of some of the viruses will be presented i.e., bovine viral diarrhea (BVD)/border disease virus (BDV), pararinfluenza-3 virus (PI-3), blue tongue virus (BTV) and respiratory syncytial virus (RSV). Diagnostic procedures and reagents are well defined for these viruses as are the availability of commercial vaccines. The importance of these viruses in respiratory tract diseases of sheep has not been clearly defined but it is generally assumed they have a causal relationship. To be contrasted with above mentioned viruses, adenoviruses present another situation. Diagnostic procedures are not well defined nor are the different antigenic strains of viruses generally available to researchers in the USA. Vaccines are not available for sheep

adenoviruses in the USA. Accordingly, a more extensive literature review will be presented for adenoviruses in order to obtain a more complete understanding of these agents in the ovine respiratory disease complex.

Historical Background

The discovery of influenza virus in 1933,[5] greatly enhanced the search for additional agents which induced respiratory diseases in humans. Respiratory diseases continued to impose substantial clinical and economical problems; disease was referred both in terms of endemic and epizootic episodes.[5] The search remained generally unsuccessful until Enders *et al.* developed tissue culture production methods which allowed the efficient *in vitro* cultivation of viruses.[52] Shortly here after, two research groups described the isolation and characterization of adenoviruses. First, Rowe and coworkers detected adenoviruses in explants of infected human adenoid tissue.[110] Secondly, Hilleman and Werner, studying an epidemic of influenza disease in army recruits, isolated several similar cytopathogenic agents from respiratory secretions added to cultures of human upper respiratory tissues.[5] As the virus was detected from the adenoid tissue, the name adenoviruses was retained in classifying these agents.[52] Following the isolation of human adenoviruses, veterinary virologists began the search for adenoviruses which infect animals. These viruses have now been detected from a wide range of animal species.[29]

Host Range

Adenoviruses are ubiquitous[58] and the number of species isolated and characterized is increasing with the passage of time. Animals for which adenoviruses are described includes cattle, horses, sheep, goat, llamas, pigs, birds, mice and reptiles.[11, 65, 87, 95] For the time being there are 9 bovine ,[21] 6 ovine, [118] 2 caprine,[89] 4 porcine, 1 equine, 1 murine, and 14 avian [93] species of adenoviruses. Many

other have been isolated but have not yet officially classified. Adenovirus infections are primarily host specific; however, cross infections can occur among closely related species. For example, lambs can be infected by bovine adenoviruses.[128]

Pathogenicity of Human Adenoviruses

There are now 41 distinct antigenic types of human adenoviruses.[29] Infection has been associated with a wide variety of disease syndromes, including conjunctivitis, pharyngitis, keratitis, bronchitis, bronchiolitis with pneumonia, cystitis and enteritis.[28, 62, 101, 121] Infection is usually mild but is much more severe in children and military recruits.[22] Likewise, different adenovirus types vary in their virulence with some strains more consistently inducing severe disease.[22]

Bovine Adenoviruses

Klein and coworkers first reported[69] the presence of an adenovirus in cattle in the USA. They isolated bovine adenovirus (BA) from the feces of an apparently healthy cow.[69] Subsequently, numerous other investigators reported the isolation of these agents from a variety of disease syndromes. Bovine adenoviruses are unique in that there appears to be two diverse virus types which infect this species. Accordingly, BA are classified into two subgroups. Subgroup one BA (species 1,2,3,and 9) possess common group-specific complement-fixating antigens with adenoviruses of all mammalian types, as well as other unique features, i.e., types of inclusion body, etc. Subgroup two BA (species 4-8) do not possess group-reactive antigens but show other unique functions. In addition, the two different subgroups of BA appear to vary in regard to their pathogenesis of infection and manner by which virus can be detected in infected animals.[88]

Bovine adenoviruses have been isolated from apparently normal animals and those exhibiting a variety of disease syndromes. Some members of BA subgroup one have been associated with enteritis and pneumonia. Subgroup two BA

frequently induce a viremia with viral localization in the respiratory and enteric tracts and corresponding signs of disease being expressed as pneumonia and enteritis.[8, 27, 90] Likewise, viremia allows access to the developing fetus and results either in fetal death or neonatal infection.[4, 30, 36, 42, 120]

Ovine Adenoviruses

Currently, six antigenic species of ovine adenoviruses (OAV) and two species of caprine adenoviruses (CAV) have been described.[89] These numbers will unquestionably increase as research continues.

Ovine adenovirus (OAV) have been isolated from numerous countries. The percentage of adult animals possessing antibodies to these agents (indicating previous infection) varies from 60 to near 100 percent.[14, 17, 18, 37, 92, 116] Ovine adenoviruses are frequently isolated from clinically normal lambs as well as from lambs with a history of enteric and respiratory disease. Further studies have shown these viruses to be etiologic agents of lamb pneumonia and diarrhea. Lambs experimentally infected with OAV show signs of pyrexia, anorexia, hyperpnea, dyspnea, conjunctivitis, cough, and diarrhea.[127] Like BA, some species of OAV have been shown to produce a viremia in the dam resulting in fetal disease.[19]

It is now apparent that interspecies transmission of BAV, OAV and CAV can occur and sheep have been shown on several occasions to be naturally infected with viruses previously classified as BAV.[18] It is also apparent that young animals appear to express signs of disease more consistently when infected with adenoviruses than do older animals. Likewise, sheep experimentally infected with OAV followed by bacterial pathogens develop more severe signs of disease and pathologic changes than when infected with either microorganism individually.[39]

Isolation and Serological Studies (OAV)

Successful attempts to isolate adenovirus from sheep were made as early as 1969 by McFerran *et al.* in Ireland.[92] On the basis of morphology, physical and serological properties, adenoviruses which were isolated from the feces of diseased sheep, suggested their possible role in producing pneumoenteritis in sheep.

Belák reported that change in sheep production practices i.e., from smaller units to larger production units, resulted in populations of inhomogeneous immunological history in which the spread of known and unknown infectious disease has been promoted by lack of protection in part of the animals.[16] Nasal discharge and fecal examination of lambs during an outbreak showed the presence of reoviruses, PI-3 virus, and adenoviruses. Further studies indicated that the major part of economic losses were due to adenoviruses which accounted for 30-40-% mortality in suckling and 10-15% in fattening lambs.

Belák and Pálfi described the involvement of adenovirus in an epidemic among fattend lambs showing clinical signs of pneumoenteritis.[16] Later, Belák *et al.* described the epizootology of a respiratory syndrome in lambs and suggested an adenovirus initiated disease.[17] The virus, OAV strain Het/3, was recovered from the nasal secretions of affected lambs.[17] The virus was later shown to be related antigenically to BAV type 2. When lambs were kept in close units, they had less natural resistance because a large number of animals are gathered from different sources with different degrees of immunity. Under these conditions, along with poor ventilation, respiratory and enteric diseases developed in sheep in a fattening facility. This research group also described the course of disease by observing that the ailment started with pyrexia and diarrhea. Two to three days following the diarrhea, signs of respiratory involvement appeared. Conjunctivitis, sneezing, coughing and nasal discharge were the commonly observed signs. Diarrhea persisted for only 7 days but the respiratory symptoms remained for another week.

As time progressed, the nasal discharge became seropurulent, marked cough and other respiratory disorders developed, all of which indicated signs of chronic infection. As is common with most viral infections, bacterial involvement was also apparent. Secondary infection was characterized by high fever, loss of appetite and forced respiration. The disease in suckling lambs resulted in heavy losses.[17]

An epizootological study of respiratory tract diseases and isolation/identification of OAV 5 and 6 was done in newly weaned lambs.[72] Lambs were housed in an isolated barn with semiconfinement conditions. Over an eight-week period of study, lambs developed respiratory disease syndrome and were separated from the sheep not showing the signs of disease. The morbidity rate amounted to 13% whereas the total mortality was 4.1%, of which 57.6% was due to pneumonia. Adenovirus was present in 36% of lung specimens with pneumonia. Virus neutralization test indicated the presence of OAV-5 and OAV-6, showing that more than one OAV serotype was present within the same flock at the time.

Dubey and Sharma reported the presence of OAV-1 in India which was isolated from the showing signs of pneumoenteritis. Nasal and fecal samples were inoculated in embryonic lamb kidney (LK) cells in order to isolate the virus. He subsequently conducted a seroprevalence survey for antibodies to OAV-1 in an Indian province. Survey showed that 4.05% (44/1086) sheep, 9.15% (26/284) goats, 16.03% (21/131) water buffaloes and 4.68% (21/448) cattle had precipitating antibodies.[44,46,48]

In a further study, Duby and Sharma showed that Sheep and goats of semiarid Rajahistan a region of India, had antibodies against OAV-1 antigen. Agar gel precipitation test (AGPT) showed that 8.62% (54/625) of sheep and 6.45% (4/62) of goats had antibodies against OAV-1. The research group observed a marked difference among exotic breeds 50% (23/46), higher crosses 33.33% (12/36) and half bred 4.79% (18/474) animals while native animals did not showed the

prevalence of antibodies to this virus.[47] In further studies, a local isolate of OAV-1, isolated from sheep showing signs of pneumoenteritis, was propagated in different cell cultures of sheep and goat origin. All the cell cultures supported the growth of the virus but there were some differences in CPE and virus yield. Lung cell cultures showed a later CPE than kidney and testicular cultures, whereas the kidney cell cultures yielded higher titers than did testicular and lung cell cultures.[49]

Using lamb kidney cultures, McFerran and coworkers[91] isolated three serologically distinct OAV from feces of sheep. Two of these three isolated strains showed cross reactivity to adenovirus group antigens. Their work suggested that adenoviruses can be recovered from both healthy and diseased animals. Detection of OAV from the feces suggested their involvement in enteric infections. Darbyshire and Pereira (1964) suggested, on the basis of gel precipitation test (a group specific test), that sheep are naturally infected with adenoviruses.[36] But apart from a preliminary report (McFerran *et al.* 1969) there was no previous report of isolation of sheep adenoviruses.[92]

Davies and Humphreys[37] described the characterization of two strains of adenovirus which were recovered from sheep in New Zealand over a period of 10 months. Samples were collected from nasal secretions, feces and lungs of dead animals and propagated in lambs testicular (LT) cultures. Two distinctly different adenovirus species were isolated, neither of which agglutinated mouse, rat, guinea pig , sheep, cattle, or human 'O' erythrocytes, but one of the isolates agglutinated chicken erythrocytes. Both strains were serologically distinct on the basis of cross neutralization test. However, their relationship to five established serotypes of ovine adenovirus was not determined.

Sharp and colleagues described a new adenovirus in sheep.[116] They showed that this new strain, 7769, was quite distinct from the three serotypes of OAV previously isolated. They were able to demonstrate that strain 7769 was not

neutralized by antisera to BAV as were the three serotypes isolated earlier. They inoculated the virus in pathogen-free lambs. It replicated and stimulated an immunological response without producing any clinical signs. This suggested that either strain 7769 was non-pathogenic or cause disease in conjunction with other agents or factors.

Sharp and Rae [116]conducted a serosurvey in Scotland to determine the presence of antibodies to ovine adenovirus species 1 to 4. They obtained serum samples from 600 healthy lambs (25 farms) with the objective of demonstrating both SN (type-specific) and precipitating antibodies (group specific) antigen. Approximately 6% of the serum showed precipitation antibodies by gel diffusion tests, but neutralizing antibodies against all OAV species were present at much higher prevalence i.e., 83%.

Rushton and Sharp[111] reported that antibodies to ovine adenovirus type 4 were present in as high as 70% of serum of sheep of all ages in Britain. Virus was recovered from 8 to 10 weeks old lambs showing the signs of pneumonia.

Adenovirus group specific antigens were detected in sheep and goats by Darbyshire in United Kingdom.[36] Serum from different animals, including sheep and goats, was tested in parallel with a human adenovirus type 5 by using the AGID test. Results showed that 1/103 sheep and 33/50 goats had precipitating antibodies against adenovirus group antigen.

Thurley and colleagues[123] described the causes of sub-clinical pneumonia in New Zealand lambs. According to their findings, besides bacterial, serum antibody titres to two adenoviruses were found. Two serological distinct viruses (later confirmed as OAV) were isolated from feces and nasal secretions of sheep.

Two previous isolates of OVA from lambs in the central USA, designated as RTS-42 and RTS-151, were classified by Adair and colleagues. Two-way cross

neutralization test with six recognized OAV species, nine BAV species and four porcine adenovirus species was performed with these two isolates. Isolate RTS-42 was identified as OAV type 5 and virus RTS-151 as OAV type 6.[1]

Two serotypes of adenoviruses were isolated from sheep in Central America. Lehmkuhl and Cutlip[78] characterized these isolates and found that isolate RTS-42 was neutralized by antiserum to ovine adenovirus (OAV) serotype 5 while another isolate, RTS-151, could not be neutralized by any of the antisera to OAV serotypes 1-5 or bovine adenovirus (BAV) 1-8. However, RTS-151 did contain the adenovirus group specific antigen as demonstrated by agar gel precipitation test. Both isolates were propagated in ovine fetal cornea (OFC) cells and all showed the typical adenovirus CPE. Further, agar gel immunodiffusion tests showed that a common antigen was shared between BAV-3 and the RTS-42 and RTS-151 isolates of OAV. Erythrocytes from different species were not agglutinated by either of the viruses isolated.[78]

In a seroprevalence and microbiological study for pneumonia in New Zealand lambs, beside describing bacterial isolation and lesions, Pfeffer *et al.* reported the isolation of adenoviruses which were neutralized by antiserum to a local untyped strain of OAV (WV 757/75).[102]

Gibbs and coworkers[56] reported the presence of adenoviruses in goats in Nigeria. While examining the mucosal scraping from the large intestine of two goats for the presence of morbillivirus, an adenovirus was present in each sample in addition to the morbillivirus. The isolates replicated in primary lamb kidney cell culture with typical adenovirus cytopathogenic effects. The isolates were confirmed to be adenovirus by electron microscopy. When the isolates were compared antigenically with recognized adenoviruses for possible cross-reactivity, neither of them could be typed as one of the nine bovine and five ovine adenoviruses. Using one way neutralization test, one isolate cross reacted with ovine

adenovirus type 2, 3 and 5 and bovine adenovirus 1, 2, and 3, while the other was related to ovine adenovirus type 2. On this basis, both isolates were considered to be different serotypes. When compared by the group specific gel precipitation test, these investigators were able to demonstrate the presence of the group-specific antigen. A serologic survey showed that 35 out of 36 (97%) goats and 23 out of 28 (82%) sheep had antibodies to the respective viruses.

An Australian group, Peet *et al.*, described the presence of adenovirus in the liver of sheep which died due to cycad poisoning.[2] Cross neutralization test was carried out with this isolate, designated as PI1537/82, with the eight species of BAV, six species of OAV and four species of porcine adenovirus. The PI1537/82 was only neutralized by antiserum to a New Zealand adenovirus type (WV 757) and BAV-7 and was not neutralized by other adenovirus antiserum tested. Also, antiserum against PI1537/82 virus neutralized the WV 757 and BAV-7. Adenovirus group -specific antigen for PI1537/82 isolate was demonstrated by cross immunofluorescence between PI1537/82 and OAV-4 and also confirmed by the reciprocal of this test i.e., OAV-4 infected cells were stained by PI1537/82 antiserum.[2]

Occurrence of natural *in utero*[19] adenoviral infection in sheep was studied by using three adenovirus strains isolated from the kidney of 174 sheep fetuses. These isolates were related antigenically to BAV-2. Neutralizing antibodies against BAV-2 were present in 20% (5/25) blood samples of sheep fetuses. This study proved that transplacental transfer of adenovirus infection is possible naturally.

Tury and coworkers[126] isolated an adenovirus strain (Het/3) from nasal discharge of lambs with respiratory disease. The isolate later was shown to be related antigenically to BA-2.

Experimental Studies (OAV)

To confirm that the Het\3 adenovirus strain was the etiological agent of the disease, Belák and coworkers inoculated 1 week old colostrum-deprived lambs with the virus.[20] The Het\3 adenovirus strain was propagated in fetal lamb kidney cultures and inoculated intratracheally and intranasally on alternate days. In addition to experimental infected sheep, some lambs were kept as contact controls. All inoculated and contact animals showed the signs of respiratory disease including cough, elevated body temperature, pneumonia and diarrhea. Virus was reisolated from the respiratory and intestinal tracts, as well as from the nasal and rectal swabs.[20] In the same study Belák and coworkers, 1975, showed that serum viral neutralization (SN) antibody titers varied from 1:8 to 1:128 in recovered sheep. These investigators concluded that virus (strain Het/3) was an important etiologic agent for ovine pneumonia and diarrhea.[20]

Experimental infection in young lambs was produced with an isolate of OAV-1 isolated in India.[45] Virus was reisolated from the nasal and rectal swabs between 3-9 days PI. No virus was recovered from any other organ or body fluid. Serum antibody appeared on day 7 PI and reached maximum on day 13 PI. Fluorescent antibody test (FAT) showed viral presence in epithelium of respiratory tract, endothelial cells of capillaries and reticulum cells of intestine.

Sharp and coworkers infected two week old lambs with strain 7769 of OAV type 4.[115] Virus was recovered from both nasal and rectal swabs for up to 9 days after exposure. Lambs developed a SN antibody titre increase which confirmed the fact that they became infected but failed to develop clinical signs of disease. While this study showed that the virus replicated in both the enteric and respiratory tracts, it was flawed in that the lambs possessed low levels of SN antibodies prior to infection which probably prevented expression of disease.

Palya and coworkers inoculated 3-week-old colostrum-deprived lambs with OAV type 1 strain GY/14.[100] The agent had previously been recovered from a natural outbreak of respiratory tract disease in lambs. The virus was inoculated by intranasal and intratracheal routes. It was recovered from nasal secretions and feces for up to 10 days post inoculation (PI). Lambs developed moderate signs of disease attributed to the alimentary and respiratory tracts. The investigators compared pathologic changes observed in lambs experimentally infected with strain GY/14 and Het/3. They concluded that GY/14 appeared to induce more severe pathologic changes.[100]

In further studies with OAV strain GY/14, Pálfi and coworkers[99] noted that the acute phase of infection was manifested by signs of respiratory disease with associated pathologic changes. In the chronic phase of disease, signs were limited to reduced growth rate, varying degrees of anorexia and intermittent pyrexia. Pathologic changes were observed in the lungs and kidneys. Virus isolation was difficult in the chronic phase of the disease as the organ involved, e.g lung explants, had to be cultured with ovine fetal cells. Lambs produced a good antibody response to the virus with the titre varying from 1:32 to 1:128 on day 17 post inoculation. The authors concluded that some lambs shed virus for prolonged periods of time for factors not completely understood.[99]

Lehmkuhl and Cutlip experimentally[76] reproduced disease in one week old, colostrum-deprived lambs infected with RTS-151. Trachea and nasal routes were used for inoculation. A mild clinical response was observed between day 4-10 PI with peak clinical signs on day 7 PI. Lambs were necropsied on different days but virus was not recovered from the tissue homogenates of intestine, feces, liver or kidney. However, nasal secretions collected in between 2-8 days PI showed the virus. Preinoculation serums were negative to RTS-151 antibodies but antibodies first appeared on day 6 PI with detectable levels on day 8 PI. The titers were >

256 from day 8 to 21 PI. Failure to reisolate the virus after 8 day of inoculation was due to appearance of virus neutralizing antibodies in the serum. Antiserum to RTS-151 was prepared in sheep and labeled with fluorescein. Lung sections, when stained with labeled antibody, showed the presence of antigen while the antigen was absent in sections of liver, kidney, and small intestine. This observation was unique because in previous studies using other isolates, workers were able to demonstrate virus in digestive and urinary tracts in addition to the respiratory tract. The authors concluded that adenovirus strain RTS-151 may represent a new strain of adenovirus both in its antigenic character and organs it infects, although the lesions it produced were similar to those as of OAV-6. The study also indicated that OAV strain RTS-151 is pathogenic to young lambs. In a follow up of the previous study[33] Cutlip and Lehmkuhl described the lesions of the disease which generally consisted ofconsolidation of lungs in lambs killed sequentially to day 21 PI.

One week old, colostrum deprived lambs were infected with strain RTS-42 (*Mastadenovirus Ovi 5*) and sacrificed on different days after inoculation until 21 days PI.[74] Virus was present in nasal secretions and lung tissues in all sheep killed between 1-6 day PI. The investigators were not able to recover the virus from any other organ including intestine. Virus neutralization antibodies first appeared on day 6 PI and were high in the serum of lambs killed on day 12 PI, but the titers had dropped in serums collected on day 21 PI. Because virus was not isolated from any organ except the lungs, the author suggested that RTS-42 is a respiratory virus. The presence of virus in feces can be explained by observation that RTS-42 is acid resistant and may have replicated in respiratory tract and passed through the digestive tract.

Lehmkuhl *et al.* experimentally inoculated[75] 10-20-days old lambs with OAV-6 or *Pasteurella haemolytica* (Ph) or OAV-6 followed by Ph 6 days PI. The group which received OAV-6 or Ph alone developed mild clinical disease after 6

and 3 days PI, respectively. Animals which were inoculated first with virus and then with bacteria, developed clinical signs of respiratory disease of greater intensity and longer duration. This observation confirmed the concept of viral/bacterial synergism in producing severe disease in animals. This concept suggests that pneumonia initiated by virus provides an environment for bacteria to localize and proliferate in lungs whereas bacteria alone are removed efficiently from the lungs by body defensive mechanisms. Virus neutralizing antibodies were detected in serum on day 6 PI and levels increased to day 15 PI. Virus was present in nasal mucosa, tracheal fluid and lung tissues between 2-8 days PI.

New Zealand investigators Davies and Humphreys experimentally infected 3 to 4 months old colostrum-deprived lambs with an untyped OAV isolate strain 757/15. The virus, which was administered intranasally and intratracheally, induced moderate clinical signs of respiratory tract disease. Pathological changes were limited to the respiratory tract. The lambs experienced a viremia as the virus was detected for up to 14 days after infection in blood.[40]

Davies and coworkers experimentally infected lambs with an untyped OAV isolate, strain WV 419/75 which was recovered from the feces of a healthy lamb.[41] The virus induced clinical signs of disease. Virus was reisolated from nasal secretions and irregularly recovered from the small intestine, kidney and lymphoid tissue. In a further study, Davies and colleagues observed the relationship and course of disease when lambs were infected with ovine adenovirus followed by *Pasteurella haemolytica* (Ph).[39] Ten to eleven week old caesarian-derived, colostrum-deprived lambs were used in the study. Various groups of lambs were given different combinations of virus or bacteria. Clinically the group which received the virus followed by the bacteria developed more severe signs of respiratory tract disease while the groups that received the virus or bacteria alone had minor signs. Similarly, reisolation of bacteria from the group inoculated with Ph alone

was low as compared to the group which received both bacteria and virus together. This showed that bacteria alone was eliminated efficiently from lungs by body defense mechanisms. This study suggested that the OAV strain WV 419/75, must be considered as another potential respiratory pathogen capable of initiating a bacterial bronchopneumonia.[39]

Respiratory Syncytial Virus (RSV)

Berthiaume *et al.* reported the presence of RS virus complement-fixating antibodies in Quebec (Canada) sheep. Survey report indicated that 81% sheep serums were positive to RSV, which was higher than bovine (14%) and horses (6%) serums.[23]

Spraker and Collins reported the presence of BRSV in Colorado bighorn sheep. Serum samples from animals showing signs of respiratory distress revealed the presence of antibody titres to BRSV and PI-3. Respiratory syncytial virus was present in tissues of the respiratory tract of sheep which died of the disease.[119]

Dunbar *et al.* reported the sereoprevalence of RSV infection in free-ranging, 6 months to 18 years old, bighorn sheep from 9 western states of USA.[50] Survey results showed that 42% (187/447) had SN antibodies to BRSV with titers ranging between 1:5 and 1:640 (median titre was 1:10).

Evermann and others[54] conducted indirect immunofluorescence and virus isolation test on sheep showing the signs of rhinitis. They revealed the presence of RSV. This isolate was neutralized by BRSV antisera and later was identified as ovine RSV. They concluded that RSV can be grouped among the viruses which cause mild respiratory problems in sheep.

Intranasal and intratracheal inoculation[26] of lambs with an ovine strain of RVS resulted in mild clinical signs of respiratory tract disease and mild

pathological changes in the lung. This virus also caused the lower respiratory tract lesions in experimentally infected calves and deer.

Lehmkuhl and coworkers successfully infected lambs with RSV of bovine origin.[80] Lambs manifested clinical illness, lesions, and serologic titers against BRSV. Virus was reisolated from nasal secretions collected 5 day PI and from tracheal fluid and lung tissue on day 7 PI. In another experiment, Lehmkuhl and Cutlip infected 6-months-old (feedlot-aged) lambs with BRSV and found the same findings as reported for young lambs.[81]

Cutlip and Lehmkuhl described the lesions in lambs infected experimentally with BRSV. The main pulmonary lesions observed were multifocal areas of consolidation with necrosis of individual epithelial cells of airways and accumulation of necrotic material in terminal airways.[35]

Trigo *et al.* inoculated lambs with BRSV via aerosol route which resulted in mild clinical response on day 4 PI. Bovine respiratory syncytial virus was reisolated from nasal secretions samples taken in between 2 to 6 day PI. No outstanding macrolesions were observed. Virus was mainly present in alveolar walls and occasionally in bronchiolar epithelium. Low levels of BRSV specific antibodies were detected in pulmonary washings.[124]

Sharma and Woldehiwet described the experimental infection of BRSV in lambs.[114] Clinical signs of disease were limited to respiratory distress i.e. dyspnoea, hyperpnoea and cough. Virus was recovered from nasal swabs in 38% (9/24) of infected lambs between 3-7 day PI. Virus was reisolated from tracheal and lung tissue of all sheep killed between 3 to 11 day PI. Bovine respiratory syncytial virus antibodies were first detected on day 6 PI with highest titers in between day 14 to 21 PI. The main pathological findings were multifocal areas of consolidation.

Trigo and coworkers studied the interaction[125] of BRSV and Ph in respiratory tract disease. Mild clinical signs were seen in lambs given virus and/or bacteria. Gross respiratory tract lesions were present in 50% (3/6) of lambs which received the virus followed by bacteria, 3 or 6 day PI of virus. The lambs which received the virus and bacteria simultaneously also had gross lesions but these were less severe than in the previous group (1/6). However, gross lesions were absent in the lambs which only received virus or bacteria alone or the group which received the bacteria first followed by virus. These results showed that viruses initiate the process and bacteria produces the severe symptoms.[125]

Al-Darraji and coworkers[6] experimentally infected lambs with RSV and *Pasteurella haemolytica* (Ph) and studied the clinical and microbiological response. Lambs inoculated with either agent or in combination manifested clinical signs but signs were more pronounced in lambs which received virus first and then bacteria. It was observed that lambs which received bacteria 5 days PI of virus were affected more severely than those that received bacteria 3 days PI of virus. About 50% (8/15) samples revealed the presence of virus and all lambs responded serologically to RSV but not to Ph. In a previous experiment,[7] these researchers demonstrated the viral antigens in bronchial and bronchiolar epithelium and in the alveolar walls by using the immunofluorescence technique. No bacterial antigen was detected.

Border Disease Virus (BDV)

The clinical effects of BD virus on fetal lambs were first described by Markson and coworkers in 1959.[85] Although the presence of the virus was not confirmed in this report, it represent the earliest clinical description. The disease was referred to as hypomyelinognesis congenita of sheep. The disease, with unknown etiology, was clinically characterized by trembling, ataxia, and shaking of head. Detailed examination revealed no gross abnormalities with negative bacteriology on brain and spinal cord specimens.[85]

Hughes *et al.* described the disease syndrome in newborn lambs and named it "B" or border disease. This disease with unknown etiology was clinically characterized by a change in birth coat, smaller lambs with retarded growth and varying degrees of trembling.[64]

Border disease of sheep is of economic importance due to persistence of viral infection which results in infertility and abortion, low survival rate, and low carcass of affected lambs. Sawyer *et al.* studied a flock of sheep in which border disease was enzootic.[112] At the start of the study 82% (125/152) ewes were seropositive to cross-reacting BVDV. Lambs born to these ewes manifested clinical signs of BD. Virus was recovered from blood lymphocytes and was shed in urine, saliva, and feces through 10 weeks of age.

Niemi *et al.* described the prevalence of BD virus in Idaho sheep.[94] It was found that 29% (73/249) were seropositive to the antigenically related togavirus, BVD virus. For neonatal lambs,51% (172/337) possessed antibodies to BVD virus. It was also observed that a significant number of BVDV seropositive lambs were derived from seronegative dams. Beside serologic evidence, BD virus was isolated from nasal and vaginal samples obtained after 24 hours of parturition.

Ames and coworkers investigated fetuses from a flock of sheep with the complaint of hairy fleece, rhythmic tremors, and unthrifty signs in young lambs.[9] Beside detecting a virus antigenically related to BVD virus, high serum titers of antibodies against the virus were present in newly born lambs. This observation indicated that *in utero* infection with border disease can occur in lambs.

Osburn and coworkers conducted the SN test on serums of lambs naturally or experimentally infected with BD virus and estimated immunoglobulin gamma content (IgG). Results showed that IgG level in precolostral control samples was less than 0.10 mg/ml while it ranged in between 0.10 to 1.1 mg/ml in infected lambs. Postcolostral values exceeded 7.5 mg/ml. Serum neutralizing antibodies to hog cholera were detected in 100% (13/13) of lambs which were infected and the level ranged between 1:4 to 1:1024. Sixty two percent of the total samples (8/13) showing antibody levels to hog cholera also had antibodies to BD virus.[76] It should be noted that BD virus and hog cholera share common antigens.

Potts and coworkers isolated BD virus from the CNS and spleen of an infected lamb.[104] The virus was propagated in tissue cultures without apparent CPE. Primary sheep choroid plexus cultures were used to develop a vaccine for use in adult healthy sheep. It was noted that there was a slow response to production of neutralizing antibodies which were detected as late as 43 days PI.

Harkness and coworkers isolated and propagated BD virus from brain and spleen fragments of lambs with the disease.[59] The virus was used to produce disease in ewes in their 35th day of gestation. Lambs born to these ewes showed varying degrees of clinical signs of border disease.

Terpstra detected Border disease antigen in tissues of sheep showing clinical signs of disease as well as in cell cultures during virus isolation studies.[122] The study showed that in young animals almost every organ was involved, as demonstrated by immunofluorescence. It was also observed that a number of organs

involved decreased with the advancement of age. Antigen was present in cultures grown from kidney, brain, and testicles, even in tissues of lambs whose sera was negative to neutralizing antibodies. The author mentioned that immunofluorescence in the CNS was usually absent or was limited to scattered neuron bodies in the cerebral cortex and cerebellum. Virus was present in the respiratory tract mainly in secretory glands of the submucosa.[122]

Anderson and coworkers experimentally produced BD in lambs by inoculating their dams at 50 days of gestation.[10] Dams showed the presence of SN antibodies to BVDV while lambs manifested the clinical signs of BD. The virus was readily detected by fluorescent antibody studies in both white and gray matter of spinal cord cells of lambs.

French *et al.* inoculated pregnant ewes intravenously (IV) with an ovine isolate of BVD virus on different days of gestation and necropsied them 15 days after the last inoculation.[55] Virus was not recovered from fetuses or fetal membranes of ewes inoculated at 14 to 16 day post mating. But virus was present in placentas and fetal membranes of sheep in a group inoculated between 36 to 119 days after mating. Highest recovery of virus was from the ewes inoculated in 36 to 38 and 60 day post mating. The researchers observed no macro or microscopic lesions in fetuses, placentas or uteri. All ewes, which were given the inocula intravenously, showed presence of SN antibodies to virus while 85% (11/13) suckling lambs also showed a titer to the virus. The titers were lower in the groups which were fed virus in food and drinking water than those which received virus intravenously. A similar situation was seen with suckling lambs.[55]

Parainfluenza virus-3 (PI-3)

Hore, D.E reported the isolation of PI-3 virus from sheep in the United Kingdom. Virus was identical to PI-3 virus isolated from cattle when considering the morphological, chemical, and serological properties of the virus.[61]

Lehmkuhl and Cutlip isolated and characterized the PI-3 virus from a lamb which died during the course of an enzootic of acute respiratory tract disease.[79]

Ditchfield reported the presence of PI-3 virus in Canadian sheep and found the same properties of the virus as described by Hore in 1966.[43]

Bluetongue Virus (BTV)

Erasmus described the importance, clinical manifestation, and economic significance of bluetongue in South African sheep and goats. Bluetongue is basically a disease of sheep and almost all breeds whether indigenous or non-indigenous are susceptible to this virus to varying degrees.[53] Clinical signs starts with high temperature which may fluctuate markedly. Hyperpnea and hyperemia of buccal and nasal mucosa can be observed which may result in salivation and frothing at the mouth. Economically, direct losses occur due to mortality but indirect losses i.e. delayed marketing, weight loss, poor body condition, and low carcass scores, are of greater economic importance.

Odiawa *et al.* conducted a serosurvey for bluetongue (BT) and epizootic hemorrhagic disease (EHDV) in ruminants in Georgia.[97] Beside cattle, deer, and goats, 34% (97/286) of sheep were seropositive to BT virus while 29% (83/286) had antibodies to EHDV. The virus was isolated from healthy sheep as well as those showing signs of disease.

Seroepidemiological Surveys (OAV, RSV, BDV, PI-3, BTV)

LeaMaster and coworkers conducted a serologic prevalence study in a flock of sheep in Idaho to determine the presence of neutralizing antibodies against bovine viral diarrhea/border disease (BVD/BD), parainfluenza type 3 (PI-3), infectious bovine rhinotrachitis (IBR) and respiratory syncytial virus (RSV). The highest percentage of antibodies was found in neonates which appeared to be of colostral origin. As the antibody titre decreased in young lambs, natural infection occurred and the prevalence of antibodies increased. Adult sheep had detectable antibody titers against all viruses except IBR. Serosurvey results from adult sheep showed the prevalence of antibodies for PI-3 to be 88% (65/74), for BVD/BD 39% (29/74) and for RSV 57% (42/74).[73]

Lehmkuhl and others conducted a two year seroepidemiological study[77] on lambs for the presence of common respiratory viruses. First serum samples were collected from 1-2 month old ram lambs and the second samples were taken two months later. Serum virus neutralization test, HI and AGD tests, appropriate to each virus, were used to determine the presence of antibodies. The mean prevalence for both years of blood collection shows; 95% for OAV-5, 87.2% for PI-3 virus, 84.5% for RSV, 41.7% for OAV-6, 8.7% for BVD virus, 5.4% for BHV-1 and 3.3% for ovine progressive pneumonia (OPP) virus. Ovine adenovirus-6 (RTS-42) had the highest infection rate on the basis of mean of two year serum analysis i.e. 42.8% (207/484) as determined by the increase of greater than 4-fold serum antibody titre from the first to sceond sample. Infection rates for other viruses were; 31.1% for OAV-5 (RTS-151), 15.3% PI-3 virus, 5.6% for RSV, 0.6% for BVD virus and 0.4% for BHV-1.

A seroprevalence survey for antibodies to seven viruses was conducted in Minnesota (USA) with healthy ewes. Three different tests, specific to each virus, were used in order to determine the level of antibodies. The result showed

presence of antibody of 0.3% for BVDV (1/377), 0.5% (2/377) for IBR, 7.6% (29/378) for BAV-3, 52.5% (200/378) to BRSV, 71.7% (273/273) for PI-3 virus, and 55% (210/379) for ovine progressive pneumonia (OPP). Serums of all sampled ewes were negative to blue tongue virus (BTV) antibodies. Presence of antibodies to adenoviruses were not included in the study.[57]

A seroprevalence survey against some viruses in Louisiana sheep was carried out by Brako and coworkers. There was a prevalence of 2.5% (4/158) for BVDV, 74.1% (117/158) for PI-3 virus, 48.7% for BRSV, and 13.3% (21/158) for BTV. Sera from all sheep was negative to BHV-1 and BLV (bovine leukemia virus).[25]

In a survey conducted in Canada by Elazhary *et al.* it was found that antibodies against the four common respiratory disease-producing viruses were present in the serums of healthy sheep and goats. It was reported that antibodies to BRSV were present in 31%, BVDV 22.2%, bovine herpesvirus-1 10.8%, and for PI-3 23.2%.[51] No age differences among adult and young animals were found in response to antibody prevalence.

A seroprevalence survey[62] against some respiratory virus in sheep and goats, in Quebec (Canada) showed that 28%, 72% and 35% of sheep and 26%, 64% and 36% of goats were seropositive to PI-3 virus, reovirus type 3 and RSV, respectively.

Adair and colleagues conducted a serosurvey[3] for antibodies to respiratory viruses in Northern Ireland. They compared non indigenous breeds of sheep to indigenous breeds in order to determine the differences in prevalence of serum antibodies against most commonly involved viruses causing respiratory problems. It was estimated that 56% (112/200) of non indigenous sheep had antibodies to PI-3 in contrast to 50% (100/200) for indigenous breeds. A marked difference was noted in the incidence of antibodies to BDV with non local sheep possessing antibody in 53% (106/200) of cases while local sheep showed 11% (22/200)

prevalence. As cited earlier, adenovirus infection is common in sheep all over the world, 89.5% (179/200) non Ireland sheep had antibodies to OAV-1 whereas 83% (163/200) Ireland sheep were seropositive to the same virus. The antibodies against OAV-2 were present in 81.5% (163/200) of non indigenous sheep while 69% (138/200) indigenous sheep had antibodies to same virus.

A pathological and microbiological survey on respiratory disease in lambs was carried out in Northern Ireland. The study lasted for two years and involved lambs 14 to 16 weeks of age. Beside bacteria, samples also showed the presence of neutralizing antibodies to RSV and PI-3 virus. Sera was negative to PI-3 on entry to the fattening facility but, after 3 weeks almost all animals showed antibodies to PI-3 virus. First year survey indicated no antibodies to RSV during a 9 week survey period. Second year serum analysis revealed the presence of antibodies to PI-3 virus at higher levels i.e. 99% (79/80) and to RSV 20% (16/80).[84] These researchers did not examine serum for evidence of antibodies to ovine adenoviruses.

Davies and coworkers conducted a longitudinal serologic study[42] by collecting monthly serum samples from four flocks of sheep of different ages in New Zealand. Serums were tested against two known viruses i.e. PI-3 and adenovirus (AV) WV 757/75. Both viruses were isolated from the sheep in New Zealand. Maternal antibodies were present in new born lambs against both viruses but they declined until all lambs were susceptible to both viral infections. All the new infections, as indicated by rising antibody titers, showed the presence of both viral infections to lambs. These investigators suggested the possible role of these viruses in causing pneumonia in sheep, although no casual relationship could be proven.

Rosadio *et al.* conducted a seroepidemiological survey in Peruvian sheep. Samples were collected from different regions of the country to determine

antibody levels and thus evidence of previous viral infection. Immunoassay for seven different viruses by specific test to each virus indicated that 47% (16/34) of sheep were positive to RSV, 82% (28/34) for PI-3 virus, 3% (1/34) for BVD/BD virus, 56% (19/34) for blue tongue virus (BTV), and 15% (5/34) for ovine progressive pneumonia (OPP) virus. No antibodies were detected to BHV-1 and bovine leukosis (BLV) virus.[108]

Loken *et al.* conducted a serological investigation[82] in ruminants in Norway. Beside other ruminants, a total of 3712 ewes (103 flocks) serums were tested for antibodies to BVD virus. The prevalence rate of seropositive ewes were 4.5% and seroreactors were found in 18% of sheep flocks. The research group noted a higher titre of antibody against the bovine virus than against the antigenically related swine fever virus.

Pommer and Schamber conducted a two year study on recently weaned lambs to determine the dynamics of antibody titre increase to PI-3 virus, RSV, and an untyped OAV. Virus isolation study for two years showed that PI-3 virus was present in 1/275 while OAV was present in 13/275. Adenovirus antibody titers varied greatly with age of the animal and stage of infection. During the period from weaning to when samples were taken at the acute phase of infection, 11.7% (14/119) had a four fold increase in serum antibody. From acute to convalescent disease period, 5.8% (7/119) showed a four-fold increase in antibody titre. Very little change was observed in PI-3 virus antibody titer range in lambs showing signs of disease during the two-years of study. Only 2.5% (3/118) lamb had a four-fold increase in antibody titers from the acute phase of disease. No appreciable change was seen in RSV antibody titers during the study. These conditions showing that RSV was not involved in causing respiratory problems.[103]

In order to determine the cause of abortion in cattle and ewes, Kirkbride and coworkers tested the serum of 994 bovine and 553 ovine aborted fetuses against

border disease (BD) virus, bluetongue (BT), bovine viral diarrhea (BVDV) virus, and leptospiral infection.[70] No antibodies were found against BT from aborted bovine and ovine fetuses. Bovine viral diarrhea virus was detected serologically in 3 of 80 aborted fetuses from ewes. While BD virus was detected in 14 of 486 fetal lambs, only 3 of 12 serums tested showed antibodies to BD virus. Study showed that these viral agents do infect the fetuses but it is very difficult to determine when they are the cause of abortion. Previously it had been demonstrated that bovine fetuses are capable of responding immunologically to these agents at some stage of pregnancy.

III. MATERIALS AND METHODS

Test Animals

This investigation was conducted during June and July, 1991 and involved three different studies. Two studies were conducted in a feedlot near Hermiston, Oregon and the third was conducted in a feedlot near Platteville, Colorado. Lambs in the Oregon studies were purchased from multiple sources in the Willamette Valley of Oregon, while lambs in the Colorado study were purchased at various locations in the state of Texas. In all cases, lambs were of mixed breeds and varied in weight from 27 to 36 Kg.

The first Oregon study was composed of 200 subjects which were chosen at random from approximately 800 lambs within two days of their arrival at the feedlot. The feedlot was feeding approximately 5000 lambs at the time. The study lambs were placed in pens by themselves, a permanent tag was affixed to their ears, and a blood sample for serological studies was taken. A second blood sample was taken from the same animals 21 days later. Lambs which died from respiratory tract disease were necropsied at the feedlot. Intact lungs and associated lymph nodes were removed and preserved with containers of frozen water and shipped to the laboratory. On arrival, samples of lungs and bronchial and mediastinal lymph nodes were removed, sections were made with a cryostat, and, the sections were examined by fluorescent microscopy for viral antigens. Serums were separated from rbcs at a local laboratory, frozen at -20C and shipped to the laboratory at the end of the trial in a frozen state.

The second Oregon study was a seroprevalence investigation for antibodies to the viruses in question. Fifty lambs which entered the feedlot from 6-15-91 to 6-18-91 were selected at random from five different pens (N=250) and blood samples for serological studies were taken. The number of lambs per pen varied from

500 to 700. A second blood sample for serological studies was taken from animals which were selected at random from the same pens 24 days later, i.e., blood samples were not paired. Serum was separated from rbcs, frozen at -20C and shipped to the laboratory while frozen.

The Colorado study was initiated on 6-3-91 and was identical to the first Oregon study and involved 200 subjects. Lambs which died of respiratory tract disease were not necropsied and samples were not examined for viral antigen. Paired serum samples from surviving lambs were separated from rbcs, frozen at -20C and shipped to the laboratory at the end of the trial.

Mediums

Mediums for growth and maintenance of cell cultures and for dilution of viruses was minimal essential medium in Earles balanced salt solution (MEM).[a] Growth and maintenance mediums contained 100 units of penicillin and 100 μg of streptomycin sulfate per ml. Growth medium was supplemented with ten percent fetal bovine serum and maintenance medium was supplemented with five percent fetal bovine serum.

Cell Cultures

Bovine turbinate cells[b] (BT) and ovine fetal turbinate cells[c] (OFTU) were propagated in plastic flasks[d] and transferred to provide additional stock cultures or used in multi-well plastic plates for virus neutralization (VN) tests.

Source of Viruses

Bovine viral diarrhea virus (BVDV),[e] respiratory syncytial virus (RSV),[f] ovine adenovirus (OAV-6),[g] and parainfluenza-3 (PI-3),[h] (Ressinger strain), were received from different sources. Bovine viral diarrhea virus, and PI-3 virus were

propagated in BT cells while OAV-6 was propagated in OFTU cells. Respiratory syncytial virus was propagated in African green monkey kidney cells (Vero cells).

Assay for Antibodies

Presence of VN antibodies to BVDV (border disease virus, BD), RSV and OAV-6 were assayed in microtiter plates.[i] Serums were heat-inactivated at 56C for 30 minutes and diluted in 2-fold steps using an initial serum dilution of 1:4. An equal volume of virus (50 µl) which contained 100 tissue culture medium infectious doses ($TCID_{50}$) was added to each serum dilution. The mixture was incubated 1 hour at 25C at which time 50 µl of growth medium containing $5x10^5$ cells was added. One drop of sterile mineral oil was placed in each well and the plates were incubated at 37C in an atmosphere of 2.5 percent CO_2. Cultures were examined for cytopathogenic effect (CPE) 5 to 7 days later. Each serum was tested in duplicate and serum end point titers were tabulated as the last (most concentrated) dilution in the series which inhibited CPE in both dilution sets of serum. Serums were considered to possess SN antibodies to virus if it inhibited CPE at 1:4 dilution. With paired serums, an animal was considered to have undergone serum conversion to a virus if it produced a 4-fold or greater antibody increase when comparing the first and second serum samples from the same animal.

Antibodies to PI-3 virus were tested by the hemagglutination-inhibition (HI) test. Serum was treated with a 25% suspension of acid-washed Kaolin. Serums were diluted in 2-fold steps in round bottom microtiter plates[j] beginning with a 1:4 dilution. An equal amount of virus (50µl) containing 8 hemagglutination units was added. After one hour incubation at 24C, 50 µl of a 0.2 percent suspension of washed bovine red blood cells (rbc) were added to each well. The plates were incubated 2 hours at 24C and examined for hemagglutination. A serum was considered to possess HI antibodies if it inhibited agglutination of rbc at a 1:8 dilution.

As with the SN test, definition of serum conversion required a 4-fold or greater antibody titer increase with paired samples.

Agar gel test immunodiffusion kits were used to measure antibodies to ovine progressive pneumonia (OPP).[k] Any evidence of a precipitation line in the agar with the test serum which joined to the positive control serum line constituted the evidence of antibodies to OPP.

The agar gel immunodiffusion (AGID) test was used to detect antibodies to bluetongue virus. Antigen and positive control serum were purchased from a commercial laboratory and the test was conducted following the vendor's directions.[l]

Fluorescent Antibody Technique (FAT)

Conjugated antiserum used to detect viral antigen to BVDV, RSV, and PI-3 were received from the OSU Veterinary Diagnostic Laboratory.[m] Antiserum to OAV-6 was prepared specially for this project. Culture flasks containing OFTU cells were inoculated with the virus and, after cell cultures developed a cytopathic effect, they were scraped from the flasks and resuspended in a saline solution. The cells were subjected to ultrasonic disruption and the preparation was clarified by centrifugation at 3000 times gravity for 20 minutes. The clarified virus suspension was subjected to isopycnic centrifugation (density 1.33 g per ml) at 100,000 times gravity for 36 hours at 4C. The adenovirus band was dialyzed against physiological buffered saline (pH 7.5) for 12 hours. The preparation was divided into four aliquots. Aliquot one was mixed with an equal volume of Freund's Complete Adjuvant and injected subcutaneously and intramuscularly in a New Zealand white rabbit. Aliquots 2-4 were frozen until they were used at which time they were thawed and mixed with an equal volume of Freund's Incomplete Adjuvant. One aliquot each was injected into the original rabbit on day 7, 14, and 21 by subcutaneous inoculation. The rabbit was exsanguinated by cardiac puncture on day 35. Serum

was separated from red blood cells and stored at -20C until it was used as positive control for SN test or to conjugate for fluorescent antibody studies.

Preparation of serum and method of conjugation with fluorescence isothiocyanate (FITC), was followed as a routine laboratory procedure.[n] Sections were reviewed with a Zeiss compound microscope using epifluorescence. Filtration of light was designed specifically for use with FITC. Sections of lung, bronchial and mediastinal lymph nodes were prepared with a cryostat and placed on a glass slide. The tissue was dried at 37C, then fixed in a 100 percent acetone solution for 20 min. After fixation, the tissue was again dried at 37C for 20 minutes. A few drops of conjugated antiserum (a different slide contained tissue for each conjugate) was placed on each tissue section. The preparations were incubated in a humidified chamber at 37C for 20 minutes after which it was rinsed with fluorescent antibody buffer (physiologic saline, M/15 phosphate buffer pH 7.6). The slide was rinsed in distilled water for 20 seconds, dried to remove water, and mounted in FA mounting solution with a coverslip. Positive control tissue (to check each conjugate) was routinely prepared. Detection of viral antigen was apparent by the appearance of focal areas of intracellular fluorescence.

Statistical Analysis

Statistical analysis was employed in the seroprevalence section of this investigation (Second Oregon Study). The significance of prevalence of antibodies to the tested viruses was evaluated by the Chi Square test.

[a] Catalogue # 410-1600EF Gibco Laboratories, Grand Island, New York.

[b] Recived from Veterinary Service Laboratory, PO Box 844 Ames, IA 50010.

[c] Received from Dr.Howard Lehmkuhl, National Animal Disease Center, PO Box 70 Ames, IA 50501.

[d] 31Catalogue # 25110-75 Corning Glass Works, Corning NY 148.

[e] Idem footnote b.

[f] Idem footnote c.

[g] Idem footnote c.

[h] R. Sweat, Fort Dodge Laboratory, Fort Dodge, IA 50501.

[i] Catalogue # 62408-305 Corning Glass Works, Corning NY 14831.

[j] Catalogue # 3910 Becton Dicknson and Company 2 Bridgewater Lane, Lincoln Park, New Jersey 07035.

[k] Idem footnote b.

[l] Catalogue # 33367 Veterinary Diagnostic technology Inc., Wheatridge CO 80033.

[m] D. Mattson, Veterinary Diagnostic Laboratory, College of Veterinary Medicine, Oregon State University, Corvallis, OR 97331.

[n] Idem footnote m.

IV. RESULTS

Fluorescent antibody examination of the 40 sheep which died of respiratory tract disease during the first Oregon study is presented (Table 1, Figure 1). Presence of RSV antigen in one or more of the tissues of the respiratory tract was highest, with 9/40 cases (22.5%), being positive. This was followed by PI-3 in 6/40 cases (15%); antigen of BDV or OAV-6 alone was not present in any case. Mixed infection involving different combinations of viruses was found in eight cases. Results showed that RSV-BDV- OAV-6 was present in 2 of these 8 samples each while a combination of RSV-OAV-6 was detected in 3 of these 8 cases. A combination of BDV-PI-3, OAV-6-PI-3 and RSV-PI-3 was present in 1 of these 8 cases each. The combination of BDV-OAV-6 was not detected. No virus was detected in 17 of 40 cases.

When tabulating the presence of virus either alone or in combination with other viruses, RSV was detected most commonly in 15 of 40 cases (37.5%). This was followed by PI-3 in 9 of 40 cases (23%), OAV-6 in 6 of 40 cases (15%) and BDV in 3 of 40 cases (7.5%).

Fluorescent antibody examination of individual tissue of the respiratory tract (lung, bronchial and mediastinal lymph nodes) and the presence of viral antigen detected in such tissue is presented (Table 2). Autolytic changes occasionally made the examination of all tissues difficult or impossible. This was particularly true with the mediastinal lymph node when autolytic changes appeared to be more extensive.

The cumulative total of lambs that died of respiratory tract disease during the first 21 days of feeding is presented (Table 3). Of the 47 deaths, 23 deaths (49%) occurred during the first four days of feeding. When viral antigen was detected during this 4-day period (12 cases) the viral antigen was RSV in 7 of the 12 cases (58 %, data not shown).

Serum samples taken from lambs when they entered the feedlot in the first Oregon study showed they varied greatly with their previous exposure to the virus in question (Table 4). Presence of antibodies to RSV was most common and found in 86 of 200 cases (43%). This was followed by OAV-6 in 65 of 200 cases (33%), PI-3 in 36 of 200 cases (18%), BTV in 13 of 200 cases (7%) and BDV in 8 of 200 cases (4%) (Table 4 and Fig 2).

Seroconversion during the first 21 days with the different viruses also varied greatly. Seroconversion was highest with PI-3 in 72 of 153 cases (47%) followed by RSV in 53 of 153 cases (35%), OAV-6 in 24 of 153 cases (16%), BDV in 7 of 153 cases (5%) and BTV in 3 of 153 cases (2%) (Table 4 and Figure 2).

The seroprevalence study (second Oregon study) showed some variation with the prevalence of antibodies as presented in the first Oregon study. Serum taken on entry to the feedlot showed that no lambs possessed antibodies to PI-3 virus. However, there was a varying degree of antibodies to the other viruses (Table 5 and Figure 3). Lambs entering the feedlot possessed antibodies most commonly to OAV-6 when they were found in 122 of 255 cases (48%). This was followed by RSV in 93 of 255 cases (36%), and BDV in 6 of 255 (2%). Prevalence of antibodies 24 days later showed a higher prevalence to all viruses (Table 5 and Figure 3). Antibodies to RSV was highest in 220 of 250 cases (88%) followed by OAV-6 in 215 of 250 cases (86%), PI-3 in 18 of 250 cases (7%) and BDV in 17 of 250 cases (7%). There was a significant increase in all cases in the number of lambs possessing antibodies at day 28 as compared to those which possessed antibodies on entry into the feedlot ($P \geq .01$).

Lambs possessed antibodies to all the test viruses on entry into the Colorado feedlot (Table 6, Figure 4). Antibody to RSV was highest and found in 49 of 200 cases (25%). This was followed by OAV-6 in 32 of 200 cases (16%), PI-3 in 22 of 200 cases (11%) BTV in 4 of 200 cases (2%) and BDV 1 of 200 cases (0.5%). Lambs underwent infection with all viruses during the succeeding 21 days as evidenced by seroconversion (Table 6

and Figure 4). Infection with PI-3 was highest with 93 of 181 cases (51%) this was followed by OAV-6 in 34 of 181 cases (19%), RSV in 30 of 181 cases (17%), BTV in 3 of 181 cases (2%), and BDV in 2 of 181 cases (1%).

Isolation of bacteria and detection of mycoplasma was only undertaken from respiratory tract samples from lambs in the first Oregon study (Table 7). Cultures from lambs with extensive autolysis when necropsied were not included in the study. *Pasteurella hemolytica* was isolated most frequently, both in lambs where no virus was detected as well as lambs where one or more of the four viruses being studied were observed by fluorescent microscopy examination (53% and 52% of cases). Other bacteria and mycoplasma species were detected from respiratory tract samples (Table 7). Due to the low number of animals where the different bacteria species were detected, data was not subjected to statistical analysis.

Table 1: Detection of viral antigen in respiratory tract tissues in lambs that died of respiratory disease (first Oregon study).

Virus(es)	Number	Percent
(A) RSV	9/40	22.5
(B) PI-3	6/40	15
(C) BDV	None	0
(D) OAV-6	None	0
(E) RSV-BDV-OAV-6	2/40	5
(F) BDV-PI-3	1/40	2.5
(G) RSV-OAV-6	3/40	7.5
(H) RSV-PI-3	1/40	2.5
(I) OAV-6-PI-3	1/40	2.5
(J) No virus detected	17/40	42.5

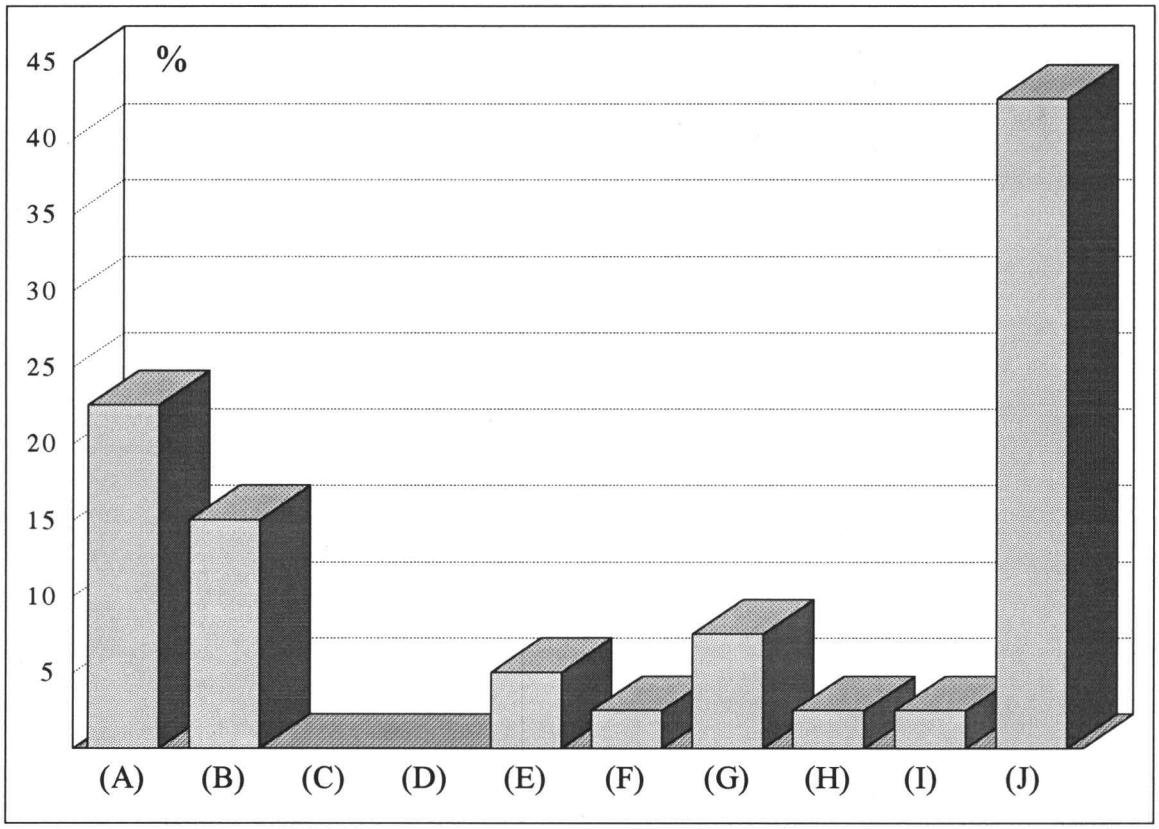

Figure 1: Percentage of positive antigen detected in the first Oregon study (see also Table 1).

(A) RSV, (B) PI-3, (C) BDV, (D) OAV-6, (E) RSV-BDV-OAV-6, (F) BDV-PI-3, (G) RSV-OAV-6, (H) RSV-PI-3, (I) OAV-6-PI-3, (J) No virus detected .

Table 2: Fluorescent antibody examination of respiratory tract tissues of sheep that died in first Oregon study.

Positive tissues	BDV	PI-3	BRSV	OAV-6
Lung	3/40	7/40	8/40	5/40
Percentage	8.00%	18.00%	20.00%	13.00%
Bronchial lymph node	2/38	7/38	9/36	4/38
Percentage	5.00%	19.00%	25.00%	11.00%
Mediastinal lymph node	0/35	3/35	11/35	3/35
Percentage	0.00%	8.50%	31.00%	9.00%

Table 3: Cumulative death of lambs which died of respiratory tract disease during the first 21 days of feeding (First Oregon Study).

Day of cumulative feeding	Autolysis; not tested for viral antigen	Viral antigen detected	Viral antigen not detected	Total cumulative deaths
1	0	5	8	13
2	1	3	1	18
3	1	2	0	21
4	0	2	0	23
5	0	0	1	24
6	0	0	1	25
7	0	1	1	27
9	0	1	0	28
11	0	2	1	31
13	0	1	1	33
16	0	0	1	34
17	0	0	1	35
18	0	0	1	36
24	1	0	0	37
25	0	0	1	38
26	0	1	0	39
27	0	1	0	40
28	1	0	0	41
32	1	0	0	42
38	0	1	0	43
39	1	0	0	44
42	0	1	0	45
44	0	1	0	46
61	0	1	0	47
Total	6	23	18	47

Table 4: Serological response of lambs to selected viruses in first Oregon study, first 21 days in feedlot.

Specimen	BDV	PI-3	RSV	OAV-6	BTV
Number with detectable antibody on entry	8/200	36/200	86/200	65/200	13/200
Percentage	4.00%	18.00%	43.00%	33.00%	13.00%
Number with seroconverted (≥ 4-fold titer increase)	7/153	72/153	53/153	24/153	3/153
Percentage	5.00%	47.00%	35.00%	16.00%	2.00%

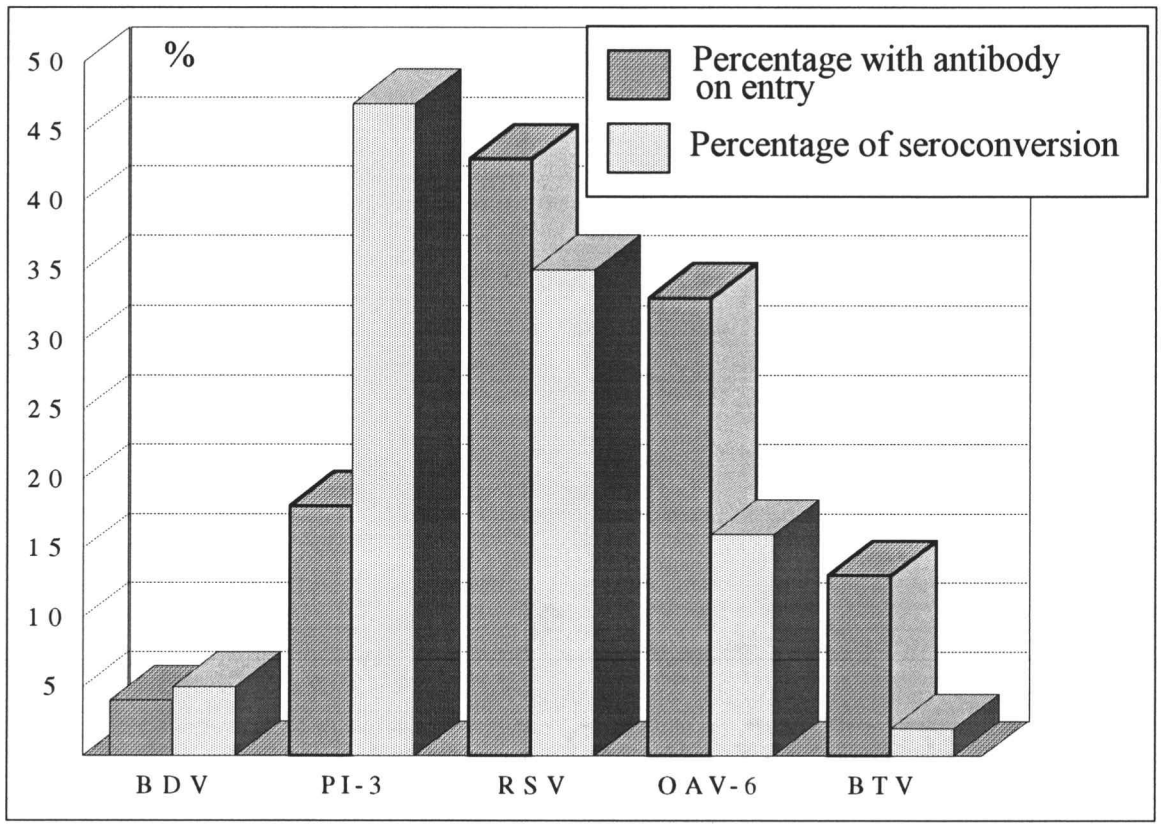

Figure 2: Percentage of serological response of lambs to selected viruses in first Oregon study, the first 21 day in feedlot (see also Table 4).

Table 5: Seroprevalence of antibodies to selected viruses in an Oregon feedlot. Samples were taken from 10 percent lambs in five pens on entry and after 24 days (Second Oregon Study).

Specimen	BDV	PI-3	RSV	OAV-6
Number with detectable antibody on entry	6/255	0/255	93/255	122/255
Percentage	2.00%	0.00%	36.00%	48.00%
Number with detectable antibodies at 24 days	17/250	18/250	220/250	215/250
Percentage	7.00%	7.00%	88.00%	86.00%

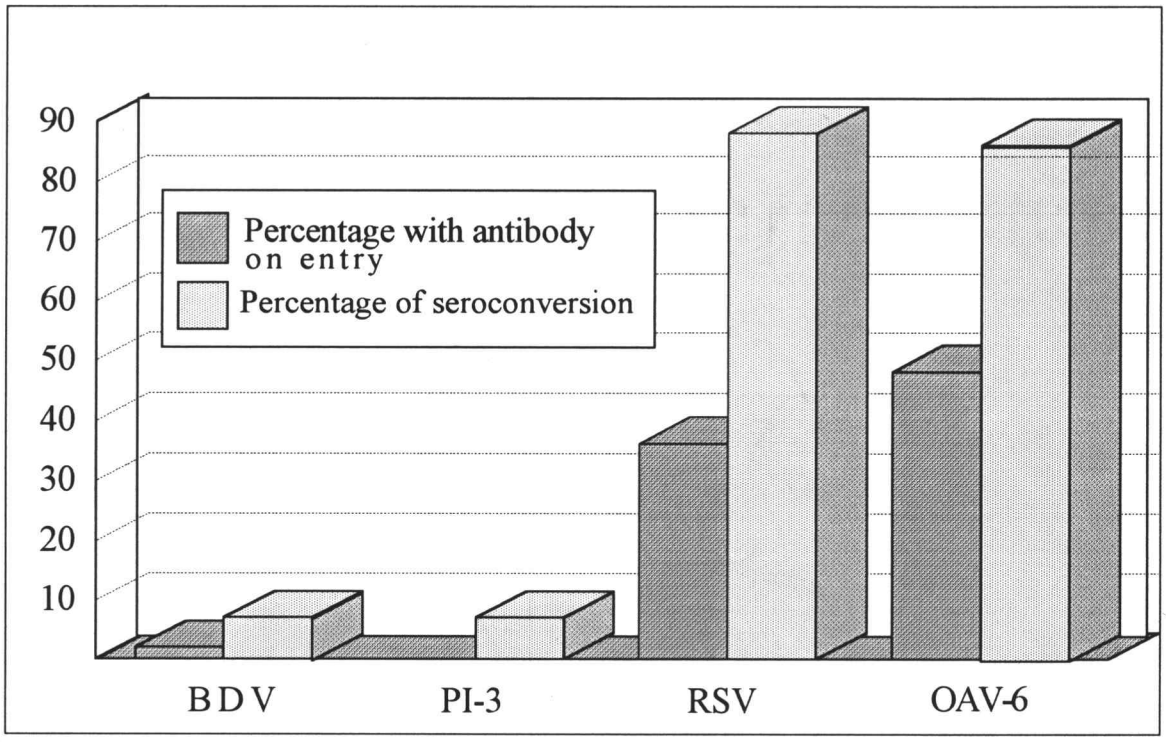

Figure 3: Percentage of seroprevalence of antibodies to selected viruses in an Oregon feedlot. Samples were taken from 10 percent of lambs in five pens on entry and after 24 days (the second Oregon study) (see also Table 5).

Table 6: Serological response of lambs to selected viruses in a Colorado feedlot, first 21 days.

	BDV	PI-3	RSV	OAV-6	BTV
Number with detectable antibody on entry	1/200	22/200	49/200	32/200	4/200
Percentage	0.50%	11.00%	25.00%	16.00%	2.00%
Number with detectable antibodies at 24 days	2/181	93/181	30/181	34/181	3/181
Percentage	1.00%	51.00%	17.00%	19.00%	2.00%

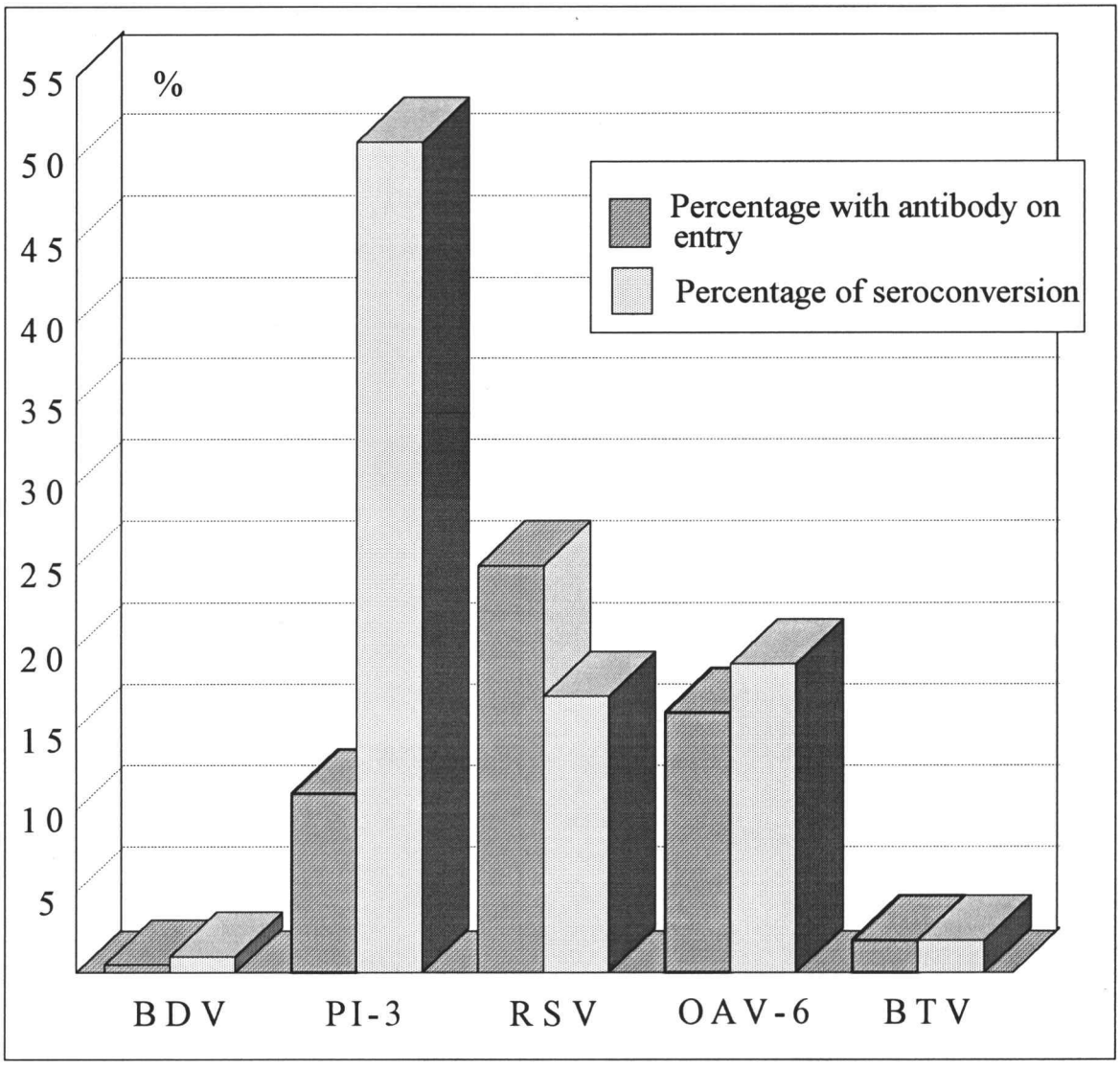

Figure 4: Percentage of serological response of lambs to selected viruses in a Colorado feedlot, first 21 day (see also Table 6).

Table 7: Detection of bacteria and mycoplasma from lambs in the first Oregon study. Data is not included from tissues where autolysis was apparent.

Type of bacteria	No virus detected	RSV	PI-3	Mixed viruses	All viruses
P. hemolytica	9/17	7/9	3/6	2/8	12/23
Percentage	53.00%	78.00%	50.00%	25.00%	52.00%
P. multocida	4/17	0/9	2/6	1/8	3/23
Percentage	24.00%	0.00%	33.00%	13.00%	13.00%
A. pyogenes	3/17	1/9	1/6	0/8	2/23
Percentage	18.00%	11.00%	17.00%	0.00%	9.00%
Mycoplasma sp.	6/17	4/9	3/6	3/8	10/23
Percentage	35.00%	44.00%	50.00%	38.00%	43.00%
E. coli	1/17	1/9	0/6	1/8	2/23
Percentage	6.00%	11.00%	0.00%	13.00%	9.00%
Staph. sp	0/17	1/9	1/6	0/8	2/23
Percentage	0.00%	11.00%	17.00%	0.00%	9.00%
Misc. bacteria	4/17	1/9	2/6	2/8	5/23
Percentage	24.00%	11.00%	33.00%	25.00%	22.00%
No bacteria isolated	0/17	0/9	0/6	1/8	1/23
Percentage	0.00%	0.00%	0.00%	13.00%	4.00%

V. DISCUSSION

This investigation was planned for the early summer months when lamb born during the preceding spring were scheduled to enter the feeding facility. In both the Oregon and Colorado feedlots, respiratory tract disease (RTD) traditionally had been a severe problem during the months of June and July.

Factors which influence RTD are multiple and believed to involve a complex interaction between infection with virus, bacteria and mycoplasma species, plus environmental factors and stress.[86, 107] The viruses under consideration in the current study are not the only ones implicated as etiologic agents for this disease problem but are considered by many investigators as the most important initiators of the disease.[60, 68, 73, 78, 79, 105]

Data from the first Oregon study showed the important association between infection and stress in initiating RTD as 49% of deaths took place during the first four days of feeding. The data from FA examination showed that, of the 12 cases where viral antigen was detected during this 4-day period, 7 of the 12 cases were positive for RSV. Infection with RSV continued over the entire feeding period as the virus spread to additional susceptible lambs. However, death rate was markedly lower after the initial period of stress. At the end of the study , a total of 15 of 40 lambs (38%) were necropsied and RSV antigen was detected in tissue of the respiratory tract.

Serologic studies confirmed the importance and prevalence of RSV infection. Serologic conversion to RSV in lambs surviving RTD (first Oregon study) was evident in 53 of 153 lambs (35%). Serologic prevalence of antibodies to RSV (second Oregon study) indicated that other lambs entering the feedlot at this time also were undergoing infection with this virus. Results showed a change from 36

percent to 88 percent prevalence of antibodies to RSV during the first 24 days of feeding.

While not as profound as the two Oregon studies, the Colorado study demonstrated that RSV infection was common in lambs. Thirty of 181 lambs (17%) which survived RTD seroconverted to the virus during the first 21 days of feeding.

In addition to the relatively frequent infection rate with RSV, PI-3 virus was detected either alone or in combination with other viruses in 9 of 40 lambs (23%) that died of RTD (First Oregon study). Serologic studies of surviving lambs confirmed a relatively high infection rate of lambs with 72 of 153 lambs (47%) seroconverting to PI-3 virus. However, other lambs entering the feedlot at this time (seroprevalence study, second Oregon study), did not experience a high rate of infection. None of the lambs entering the feedlot possessed antibodies to PI-3. After 24 day of feeding,, 18 of 250 lambs (7%) had detectable antibodies to this virus. To be contrasted with this, 93 of 181 surviving lambs (51%) in the Colorado study seroconverted to PI-3.

Infection with PI-3 in lambs may be sporadic and not predictable in regard to rate of infection. Lehmkuhl et al, conducted a serologic survey for antibodies to selected viruses involved in RTD of lambs in a ram testing station in Iowa.[77] Ram lambs (1 to 2 months old) were sampled as they entered the feeding establishment and a second sample was taken 60 days later. Infection rate of lambs was determined by seroconversion. During the first year of the study no lambs (N=236) became infected with PI-3. However, a thirty percent seroconversion rate to the virus (N=248) took place during the second year of study.

As with RSV and PI-3, Ovine adenovirus has been associated with respiratory tract disease in sheep. The OAV-6 virus was isolated originally from the lungs of lamb with pneumonia.[7, 77] In the current study, OAV-6 antigen was detected by FA in 6 of 40 (15%) lambs that died of RTD. From the same groups of lambs that

survived, 24 of 154 lambs (16%) seroconverted to the virus during their first 21 days in the feedlot.

Seroprevalence of antibodies to OAV-6 from representative lots of lambs in the feedlot at the same time (second Oregon study) also indicate that infection with this virus was common. While paired samples were not taken, 48 % of lambs (N=255) possessed antibodies at the beginning of the study. This was significantly lower than the 86% prevalence of antibody levels in lambs (N=250) at the end of the 24 day feeding period ($p \leq .01$). Lambs in the Colorado feedlot also showed serologic evidence of infection with OAV-6 with 34 of 181 (19%) seroconverting to the virus during the first 21 days.

Rate of infection with OAV-6 in the current investigation agrees with the results of a study conducted by Lehmkuhl and coworkers regarding incidence of infection with the virus in young lambs. In a seroprevalence trial of lambs at a ram testing station cited previously,[77] serum samples were taken from lambs (ages 1 to 2 months) as they entered the station and again 60 days later. Seroconversion to OAV-6 in the first year study was 51% (N=234) while rate of seroconversion during the second year was 35 % (N=250). Further studies by several investigators have shown that OAV-6 alone produces minimal pathogenic changes when injected in specific pathogen-free lambs. Disease and pathological changes were much more severe when lambs were infected with OAV-6 followed in 2-4 days by *Pasteurella hemolytica.*[7, 125]

There was a special interest in the current study concerning rate of infection with BDV. A closely related virus, both in antigenic structure and pathogenesis of infection, is bovine viral diarrhea virus (BVDV).[60] Bovine viral diarrhea virus is immunosuppressive and replicates extensively in lymphocytes, macrophage and blood platelets. In addition, BVDV replicates in mucus membrane cells of respiratory and intestinal tracts.[106] Most infections occur while young calves and lambs

still retain some colostral antibodies against the virus and an infection is usually mild. However, during periods of stress and appropriate infection with other viruses and bacteria, infection may be severe.[12, 109] In the current study, BDV antigen was detected by FA in 3 of 40 lambs (8%) in the first Oregon study. Likewise, paired serum samples of lambs which survived RTD in the same pen showed that only 5% seroconverted to the virus. Seroprevalence of antibodies from lambs in other pens in the feedlot at the same time showed a change of prevalence from 2% (N=255) to 7% (N=250) after 24 days in the feedlot. The study in a Colorado feedlot again confirmed the relatively low rate of infection with this virus with a 1% seroconversion during the first 21 days of feeding.

Results concerning the rate of infection with BDV is consistent also with the previous study by Lehmkuhl *et al.* where performance of 1 to 2 months old lambs were studied in a ram testing station.[77] After 60 days in the feeding trial, seroconversion to BDV was 0.4% the first year (N=234) and 0.8% (N=250) the second year. While these two investigations i. e, Lehmukuhl *et al.* and the current investigation are limited in scope, they are the only significant studies to date conducted with BDV in growing feeder lambs in the USA. Until further trials prove otherwise, it is felt that BDV may not play an important role in respiratory diseases of the young feeder lamb.

Ovine progressive pneumonia is a difficult disease to study using the methods in the current investigation. The virus induces chronic and progressive lung changes and predisposes sheep to other respiratory pathogens. Seroconversion (presence of antibody where previously antibody was not present) follows infection by 1 to 3 months.[32] It is very difficult to isolate the virus and traditionally infection is confirmed by the detection of antibody.[31] Assay for antibodies to OPP was tested only in lambs in the first Oregon study and no antibodies to the virus were detected. These results were expected as infection with resultant lung

changes and presence of antibody is usually observed in sheep over two years of age.[63]

Infection with BTV does not lead to respiratory tract disease but to a generalized inflammatory response due to the fact that virus replicates in endothelial cells of the vascular system.[83] Infection with BVT predisposes to stress and death. The presence of infection was studied to determine if the virus was influencing rate of mortality. The virus is difficult to detect and infection is more efficiently determined by the presence of antibody using the agar gel immunodiffusion test.[67] Infection rate is quite variable and is influenced by complex factors such as herd immunity, presence of infected carrier animals in the area and presence of a high concentration of the vector *Culicoides veripennis*.[24] Once sheep are infected, antibodies, as determined by AGID test, may take up to three weeks to reach detectable levels.[66] Seroconversion to BT virus in the first Oregon study indicated 2% of lambs developed antibodies to the virus during the first 21 days of feeding. The Colorado study likewise showed a 2% seroconversion rate to the virus. However, antibodies were present in Oregon lambs (7%) and Colorado lambs (22%) when they entered the feedlot. Lambs may have been infected prior to entering the lot and they seroconverted during the 21-day test period. Since active infection may have taken place up to three weeks previously and seroconversion was relatively low, it is felt that active BT virus infection probably did not predispose animals to the high mortality rate observed in this study.

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