AN ABSTRACT OF THE THESIS OF

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Donald E.Mattson

Six 3 to 4 weeks old, cesarian-derived lambs were inoculated with ovine an adenovirus isolate 475N. Inoculated lambs showed moderate clinical signs of respiratory distress, conjunctivitis, and loose feces during the 10-day observation period. Virus was detected from nasal and conjunctival swabs starting on postinoculation day (PID) 2. Virus was detected in the feces in a inconsistent fashion. At necropsy, virus was present in the lung, tonsils, and bronchial and mediastinal lymph nodes of lambs necropsied on PID 5 and 7. Tissue samples from gastrointestinal tract and kidney were negative for the virus. Presence of virus in the feces was believed to be from replication in tonsillar tissue. At necropsy, lambs showed signs of pneumonia and numerous intranuclear inclusion bodies were detected in affected lung tissue. Virus neutralizing antibodies appeared at low levels in serum on PID 6 and reached higher levels by PID 10.

Six ovine adenovirus prototype species, three uncharacterized ovine and bovine adenoviruses isolates and two uncharacterized llama adenoviruses isolates were digested with four different restriction enzymes. Digested viral DNA was separated in 0.7% agarose gels. The enzymes *Bam* HI, *Eco* RI, *Hind* III, and *Pst* I digested viral DNA and produced 2-10 bands. The profile of the band distribution permitted the differentiation

of the viruses under study. However, further studies using multiple isolates of each species are required to determine if this procedure will efficiently distinguish different species of ruminant adenoviruses.

Ten adenoviruses from sheep (including the six prototype species), one from bovine and one from llama were studied by virus neutralization test to determine their degree of antigenic similarities. Reciprocal virus neutralization tests were performed and the degree of antigenic similarities, i.e., strain differentiation was determined by criteria established by the International Committee for the Nomenclature of Viruses. Isolates 32CN (a bovine adenovirus) and 475N (an ovine adenovirus) were antigenically identical and not neutralized by any of the prototype species antiserum. They are candidates for a new species of ruminant adenoviruses. Ovine adenovirus isolate 47F was shown to be a member of OAV-5 species while the llama adenovirus strain represents a newly recognized species for this animal.

Characterization of Adenovirus Isolated from Sheep in Oregon

by

Shakeel Babar

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Shakeel Babar, Author

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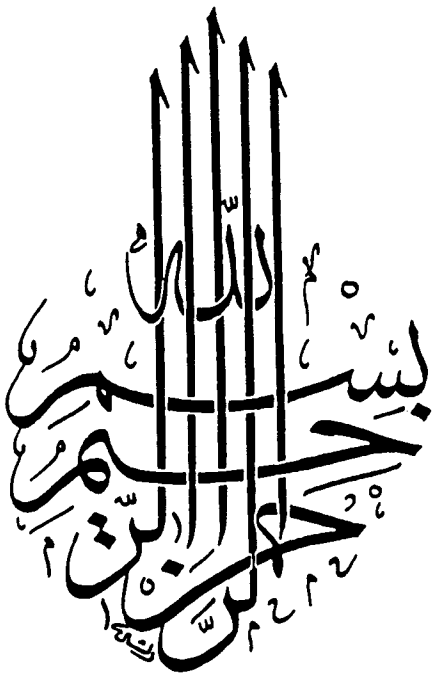
Dedicated To The Memory of My Father

Rao Babar Ali Khan

CONTRIBUTION OF AUTHORS

Dr. Stanley Snyder, Pathologist, Veterinary Diagnostic Laboratory, was involved in the necropsy of animals. He assisted by describing pathological and histopathology in necropsied animals. Dr. James Andreasen, Virologist, College of Veterinary Medicine, helped in DNA restriction endonuclease studies of viruses. He constantly provided the guidance in the experiment. Mr. Rocky Baker, Microbiologist, Veterinary Diagnostic Laboratory, helped in all laboratory procedures.



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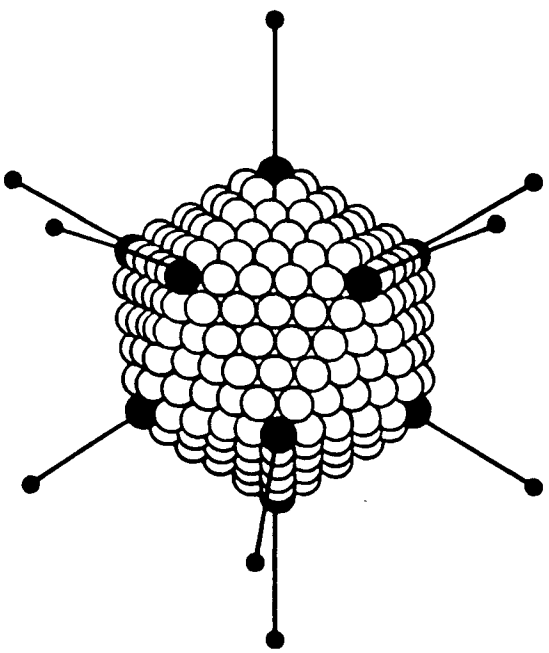
the Beneficent, the Merciful



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CHAPTER I

GENERAL INTRODUCTION

Diseases affecting the respiratory tract of sheep and goats are one of the most important factors which limit production of these species on a world-wide basis. While small ruminant production is practiced throughout the world, the economic significance of this industry is more important in some countries than others. The author of this thesis is a citizen of Pakistan where sheep and goat production is the main agriculture commodity of the country. After training in the USA, the author will return to his country where he will be involved in developing biologics for prevention of diseases of sheep and goats. Livestock production is the principal occupation of the inhabitants of Balochistan (PAKISTAN). 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 the total country's sheep and goats respectively. Overall productivity of these animals is low due to poor feeding, management factors 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 situation predisposes the animals to various infectious diseases.

The economic consequences of this situation are profound and includes 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.[58]

Respiratory tract disease in sheep is a complex syndrome caused by a number of infectious microorganisms plus the interaction of environmental, nutritional, and other poorly defined factors.[58] While several microbiological agents can initiate this disease, viruses are generally regarded as the most important etiologic agents. In order to further study the role of viruses in this complex disease situation, several approaches are appropriate. The virus in question must be characterized and diagnostic procedures should be developed. In order to determine the prevalence of the agent, a serological survey as well as virus isolation studies are of value. Using these methodologies, role of individual viruses as well as groups of viruses which naturally infect an animal can be determined. Subsequently, a more detailed investigation can then be undertaken to define the significance of each virus which has been shown to infect the species in question. These more detailed studies may involve serologic response to the 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 the virus into animals and determining the pathogenesis of infection.

An ovine adenovirus, designated as 475N was isolated from a lamb with pneumonia in 1981 in Oregon. Initial infectivity trial, pathological and serological studies showed that the agent was widespread and suggested it was involved in causing

pneumonia in lambs.[67] The purpose of this thesis is to further characterize and classify ovine adenovirus isolate 475N. This research will be divided into three parts: 1) Lamb infectivity with isolate 475N, 2) antigenic characterization of the isolate and, 3) restriction endonuclease profile of the virus.

Historical Background:

The discovery of influenza virus in 1933[24] greatly enhanced the search for additional agents which induced respiratory diseases in humans. Respiratory diseases continued to impose huge clinical, and economical problems; disease syndromes were referred to both in terms of endemic and epizootic episodes.[24] The search remained generally unsuccessful until Enders *et al.*, developed tissue culture production methods which allowed the efficient *in vitro* cultivation of viruses.[31] Shortly thereafter, two research groups described the isolation and characterization of adenoviruses. First, Rowe and coworkers detected adenoviruses in explants of infected human adenoid tissue.[59] Secondly, Hilleman and Werner, studying an epidemic of influenza disease in army recruits, isolated several similar cytopathogenic agents when respiratory secretions were added to cultures of human upper respiratory tissues.[24] As the virus was detected from the adenoid tissue, the name adenoviruses was retained in classifying these agents.[31] 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.[33]

Host Range:

Adenovirus are ubiquitous[34] and the number of types isolated and characterized is increasing with the passage of time. Animals for which adenoviruses are described includes cattle, horses, sheep, goat, pigs, dogs, monkey, birds, mice, reptiles[4, 36, 52] and llamas.[46] Currently there are 10 bovine, 6 ovine, 1 caprine, 4 porcine, 1 equine, 2 canine, 2 murine, 27 simian and 12 avian species of adenoviruses.[33] Many others have been isolated but have not yet been 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.[66]

Pathogenicity of Human Adenoviruses:

There are now 47 distinct antigenic types of human adenoviruses.[33] Infection has been associated with a wide variety of disease syndrome, including conjunctivitis, pharyngitis, keratitis, bronchitis, bronchiolitis with pneumonia, cystitis and enteritis.[16, 35, 55, 63] Infection is usually mild but is much more severe in children and military recruits.[13] Likewise, different adenovirus types vary in their virulence with some strains more consistently inducing severe disease.[35]

Bovine Adenoviruses:

Klein and coworkers first reported the isolation of a bovine adenovirus (BAV) from the feces of an apparently healthy cow.[38] Subsequently, numerous other investigators described 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.[5] Accordingly, BAV are classified into two subgroups. Subgroup one BAV (types 1,2,3,and 9) possess common group-specific complement-fixing antigens with adenoviruses of all mammalian species, as well as other unique features, i.e., types of inclusion body, etc. Subgroup two BAV (types 4-8) do not possess group-reactive antigens but show other unique functions. In addition, the two different subgroups of BAV appear to vary in regard to their pathogenesis of infection and manner by which virus can be detected in infected animals.[47]

Bovine adenoviruses (BAV) have been isolated from apparently normal animals and those exhibiting a variety of disease syndromes. Some members of BAV subgroup one have been associated with enteritis and pneumonia. Subgroup two BAV 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, 15, 48] Likewise, viremia allows access to the developing fetus and results either in fetal death or neonatal infection.[17, 19, 25, 37, 62]

Ovine Adenoviruses:

Currently, six antigenic species of ovine adenoviruses[33] (OAV) and one species of caprine adenoviruses (CAV) have been described.[49] These numbers will unquestionably increase as research continues.

The first isolation of ovine adenovirus was reported in 1969 from Northern Ireland. Three virus types were recovered from feces of clinically normal and diseased lambs. Investigators designated them as ovine adenovirus types 1, 2, and 3 (OAV-1, OAV-2, OAV-3).[51] Later, an additional ovine adenovirus type 4 (OAV-4) was isolated

from a group of lambs suffering with respiratory distress.[61] Ovine adenovirus type 5 (OAV-5) was isolated from healthy lambs in Turkey.[6] Ovine adenovirus type 6 (OAV-6) was recovered from lambs with respiratory disease in New Zealand.[20] The listing of the prototype OAV, strain designation, and original reference is shown (Table 1.1).

Table 1.1. The designated prototype strains of ovine adenoviruses showing strain designation and reference of original description.

S#	OAV Types	Strain #	Reference
1	OAV-1	SI	McFerran JB et.al., 1969, Ireland.
2	OAV-2	PX515	McFerran JB et.al., 1969, Ireland.
3	OAV-3	PX611	McFerran JB et.al., 1969, Ireland.
4	OAV-4	7769	Sharp JM et.al., 1974, Scotland.
5	OAV-5	SAV	Bauer K et.al., 1975, Turkey.
6	OAV-6	WV419	Davies DH et.al., 1976, New Zealand.
7	OAV-5 (US)	RTS-42	Lehmkuhl HD et.al., 1982, USA.
8	OAV-6 (US)	RTS-151	Lehmkuhl HD et.al., 1982, USA.

Ovine adenoviruses 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.[6, 9, 10, 20, 51, 61] 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.[13, 24, 25, 26, 28, 41, 43, 44, 60, 67] Like BAV, some types of OAV have been shown to produce a viremia in the dam resulting in fetal disease.[11]

It is now apparent that interspecies transmission of BAV and OAV can occur and sheep have been shown on several occasions to be naturally infected with viruses previously classified as BAV.[10] 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.[21, 24, 43]

CHAPTER II

Experimental Infection of Lambs with Ovine Adenovirus Isolate 475N: Clinical, Pathological, Virological, and Serological Responses.

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ABSTRACT

Six 3 to 4 weeks old, cesarian-derived lambs were inoculated with an untyped isolate of ovine adenovirus (strain 475N). Inoculated lambs showed moderate clinical signs of respiratory distress, mild conjunctivitis, and loose feces during the 10-day observation period. Virus was detected from nasal and conjunctival swabs starting post-inoculation day (PID) 2. Virus was shed from the intestinal tract starting PID 5 until the end of the trial. Virus was demonstrated both by fluorescent antibody technique and virus isolation from lung tissue, tonsils, and bronchial and mediastinal lymph nodes of lambs necropsied on PID 5 and 7. Tissue samples from gastrointestinal tract and kidneys were negative for the virus, suggestive of its predilection for the respiratory tract. At necropsy, lambs showed signs of pneumonia, while histopathological examination revealed presence of large intranuclear inclusion bodies in affected lung tissue. Antibodies to the virus appeared at low levels in serum on PID 6 and reach higher levels by PID 10.

INTRODUCTION

Diseases affecting the respiratory system of sheep are of major economic significance for this industry. Losses from this disease contribute more to morbidity and mortality in this species than does any other infectious diseases.[14, 58] Comparatively little is known concerning respiratory and enteric diseases of newborn lambs because very little research emphasis has been placed on this subject. This probably is due to the low market value for sheep and it is more difficult to sample these animals due to

managemental practices that prevail in this industry. Traditionally, the ewes and newborn lambs are held in confinement for approximately one week and then placed in a pasture. When in a group or flock setting, diseased lambs are difficult to observe and to take samples for viral isolation or serological studies. Enteric and respiratory diseases are usually experienced during first 7-21 days of the life of the animal at which time the logistic and economic factors frequently preclude taking samples.

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.[58] 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, role of 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 species 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 experimentally inoculating virus into a group of animals and determining the pathogenesis of infection.

The purpose of this investigation was to further characterize the significance of ovine adenovirus isolate 475N in the respiratory disease complex of lambs. The virus was experimentally inoculated into young lambs and its pathogenesis of infection and ability to induce disease was characterized.

LITERATURE REVIEW

Adenoviruses have been isolated from almost all domestic animals, birds, and reptiles. Although their role in respiratory tract disease is not fully understood, their involvement in this syndrome is well described. These viruses have been isolated from healthy animals as well as those showing signs of respiratory or enteric tract disease or a combination of both. In attempting to define the role of adenovirus in respiratory tract disease (RTD), serologic prevalence as well as virus isolation studies have been used. When appropriate, investigators have attempted to reproduce the disease by experimentally inoculating lambs with a specific isolate.

Using lamb kidney cultures, McFerran and coworkers[50] isolated three serologically distinct OAV from feces of sheep. Two of these three isolated strains showed cross reactivity to adenovirus group antigens. Their work first indicated that adenoviruses can be recovered from both healthy and diseased sheep. Detection of OAV from the feces suggested their involvement in enteric infections. Previously, Darbyshire and Pereira (1964) proposed, on the basis of gel precipitation test (a group specific test), that sheep were naturally-infected with these viruses.[19] But apart from a preliminary report, there was no previous report of the isolation of sheep adenoviruses.[51]

Belàk et.al., described the epizootology of a respiratory syndrome in lambs and showed that adenovirus initiated the disease.[9] The virus, OAV strain Het/3, was recovered from the nasal secretions of affected lambs. The virus was later shown to be related antigenically to bovine adenovirus (BAV) type 2. When lambs were kept in closely confined conditions, they had less natural resistance against infection because a large number of animals were gathered from different sources with different degrees of

immunity. Under these conditions, along with poor ventilation, respiratory and enteric disease spread rapidly in the sheep 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 commonly observed signs. Diarrhea persisted for only 7 days but the respiratory signs 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 death losses.[9, 66]

To confirm that the Het/3 adenovirus strain could induce respiratory tract disease, Belàk and coworkers inoculated 1-week old colostrum-deprived lambs with the virus.[12] Virus was inoculated intratracheally and intranasally on alternate days. In addition, some lambs were kept as contact controls. All inoculated and contact animals showed the signs of respiratory tract 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.[12] In the same study, these investigators showed that the viral neutralizing (VN) antibody titer varied from 1:8 to 1:128 in recovered sheep. They concluded that this virus (strain Het/3) was an important etiologic agent for ovine pneumonia and diarrhea.

Palya and coworkers inoculated 3-week old colostrum-deprived lambs with OAV type 1 strain GY/14.[54] The agent had previously been recovered from a natural outbreak of respiratory disease in lambs. The virus was inoculated by intranasal and intratracheal routes and, it was recovered from nasal secretions and feces for up to 10 days post inoculation (PI). Lambs developed moderate signs of disease attributed to infection of the alimentary and respiratory tracts. The investigators compared pathologic and clinical signs observed in lambs experimentally infected with strain GY/14 and Het/3. They concluded that GY/14 appeared to induce more severe pathologic changes.[54]

In further experimental lamb inoculation studies with OAV strain GY/14, Pàlfi and coworkers noted that the acute phase of infection was manifested by signs of respiratory disease and associated pathologic changes. In the chronic phase of the 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 lung explants had to be co-cultured with ovine fetal cells. Lambs produced a good antibody response to the virus with the titers 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 due to factors not completely understood.[53]

Dubey and Sharma[27, 28, 29, 30] characterized the properties of OAV-1 in India. The virus was isolated from sheep showing signs of pneumoenteritis. Nasal and fecal samples were inoculated on embryonic lamb kidney (LK) cells in order to isolate the virus. The virus appeared to infect a variety of animal species as sheep (4.05%), goats (7.25%) and cattle (9.15%) showed VN antibody titres against the isolate. Experimental infection in

young lambs was produced with the OAV-1 isolate.[26] 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. Virus neutralizing antibodies appeared on day 7 PI and reached a 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 the intestine.

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

An epizootiological study was conducted to determine the role of OVA-5 and 6 in RTD in recently weaned lambs.[41] Lambs were housed in an isolated barn under semiconfinement conditions. The morbidity rate was 13% whereas the total mortality was 4.1% of which 57.6% was due to pneumonia. Adenovirus was demonstrated in the 36% lungs of lambs that died of pneumonia. Virus neutralization test indicated that the lambs were experiencing infection with OAV-5 and OAV-6 during the test period.

Thurley and colleagues[64] described the causes of pneumonia in New Zealand lambs. An increase in serum antibody titre was found against two adenoviruses. These two serological distinct viruses (later confirmed as OAV) were isolated from feces and nasal secretions from infected lambs.

Sharp and colleagues described a new strain of ovine adenovirus.[61] The isolate strain (7769) was quite distinct from the three serotype of OAV previously isolated. They demonstrated that strain 7769 was not neutralized by antisera to BAV and to the three other previously isolated OAV strains. In further studies they inoculated the virus in pathogen-free lambs. The virus replicated and stimulated an immunological response without producing any clinical signs. This suggested that either strain 7769 was nonpathogenic, caused disease in-conjunction with other agents or environmental or some stress-inducing factors have predisposed the lambs to disease.

Sharp and coworkers subsequently classified 7769 as OAV-4 and infected two-week old lambs with the virus.[60] Virus was recovered from both nasal and rectal swabs for up to 9 days after exposure. Lambs developed a VN antibody titre increase (mean titre 1:10 to 1:708) 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 VN antibodies prior to infection which probably prevented expression of disease.

In a seroprevalence and microbiological study of pneumonia in New Zealand lambs, beside describing bacterial isolation and lesions, Pfeffer *et al.*, reported the

isolation of adenoviruses.[50] The isolates were neutralized by antiserum to a local untyped strain of OAV (WV 757/75), which was recovered from New Zealand.

New Zealand investigators Davies and Humphreys experimentally infected 3 to 4-months old colostrum-deprived lambs with the untyped OAV isolate (strain 757/75). The virus, which was administered intranasally and intratracheally, induced moderate clinical signs of respiratory tract disease. The lambs experienced a viremia as the virus was detected in blood for up to 14 days after inoculation.[23]

Davies and Humphreys[20] characterized two strains of adenovirus which were recovered from sheep in New Zealand. Samples were collected from nasal secretions, feces and lungs of dead animals and propagated in lamb testicular (LT) cultures. Two distinctly different adenovirus types were isolated. It was found that both strains were serologically distinct on the basis of cross VN test. However, their relationship to the five established serotypes of ovine adenovirus recognized at that time was not determined.

In an extensive study, Davies and colleagues observed the relationship and course of disease when lambs were infected with ovine adenovirus followed by *Pasteurella haemolytica* (Ph).[21] Two to four 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 virus followed by 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 were 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 bacterial bronchopneumonia.[21]

In further studies, Davies and coworkers experimentally infected lambs with one of the untyped OAV isolates (strain WV 419/75) which was recovered from the feces of a healthy lamb. The virus induced clinical signs of respiratory and enteric tract disease. Virus was reisolated from nasal secretions and irregularly recovered from the small intestine, kidney and lymphoid tissue.[23]

Two serotype of adenoviruses were isolated from sheep in Iowa. Lehmkuhl and Cutlip[43] characterized these isolates and found that isolate RTS-42 was neutralized by antiserum to ovine adenovirus (OAV) type 5 while another isolate, RTS-151, could not be neutralized by any of the antisera to OAV serotype 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.[43]

The two previous isolated strains of OAV, designated as RTS-42 and RTS-151 were classified by Adair and colleagues.[1] Two-way cross neutralization tests with six recognized OAV species, nine BAV species and four porcine adenovirus species were performed on these two isolates. Isolate RTS-42 was identified as OAV type 5 and virus RTS-151 as OAV type 6, although the serologic crossmatching was largely one sided.[1]

Lehmkuhl and Cutlip experimentally[45] infected one-week old, colostrum-deprived lambs infected with RTS-151. Tracheal and nasal routes were used for inoculation. Mild clinical response was observed between PID 4-10 with peak clinical signs on PID 7. 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 between PID 2-8 showed the presence of virus. Preinoculation serums were negative for antibodies, antibodies first appeared on PID 6 with detectable levels on PID 8. The titre were > 1:256 from PID 8-21. Failure to reisolate the virus after eight day was due to appearance of VN antibodies in the serum. Antiserum to RTS-151 was prepared in rabbits and labeled with fluorescein. Lung sections were stained with labeled antibody and showed the presence of the virus. Virus 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. In this study, an interesting observation showed that this virus was acid labile, contrary to the other five OAV prototypes (pH resistant). The investigators suggested that this might be the reason that no enteric tract involvement signs were observed. The authors concluded that adenovirus strain RTS-151 may represent a new strain of adenovirus both in its antigenic character and the organs it infects, although the lesions it produced were similar to those of OAV-6. The study also indicated that OAV strain, RTS-151, is pathogenic to young lambs.[45] In a follow up of the previous study[18], Cutlip and Lehmkuhl described the lesions of the disease. The main lesion observed was consolidation of lungs of lambs killed sequentially to PID 21.

One-week old colostrum-deprived lambs were infected with the strain RTS-42 and necropsied on different days after inoculation untill PID 21.[42] Virus was present in nasal secretions and lung tissues of all lambs necropsied PID 1-6. They were not able to recover the virus from any other organ, including the intestine. Virus-neutralizing antibodies first appeared on PID 6 and were high in the serum of lambs killed on PID 12 PI, but the titer had dropped in serums collected on PID 21. Because virus was not isolated from any organ except the lungs, the author suggested that RTS-42 induces respiratory tract disease. The presence of virus in feces can be explained by the fact that RTS-42 is acid resistant and may have replicated in the respiratory tract and passed through the digestive tract.

Lehmkuhl et.al., experimentally inoculated[44] 10-20 day-old lambs with an 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 PID 3-6 respectively. Animals which were inoculated first by virus and then by bacteria developed clinical signs of respiratory disease of greater intensity and for 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 PID 6 and levels increased by PID 15. Virus was present in nasal mucosa, tracheal fluid and lung tissues between PID 2-8.

MATERIALS AND METHODS

Test Animals and Experimental Procedures:

This study was conducted during spring of 1993 at Oregon State University, College of Veterinary Medicine in the class III animal isolation facility. These lambs were held in a pen by themselves prior to the study. Six 3-4 week old, cesarian-derived lambs were used for this experiment. Each of six lambs was inoculated with virus, intraocularly ($1.3x10^4$ $TCID_{50}$), intranasally ($2.6x10^4$ $TCID_{50}$), and transtracheally ($6.4x10^4$ $TCID_{50}$). Inoculated lambs were observed daily for clinical response. Lung auscultation, rectal temperature, and respiratory rate were recorded daily. Fecal and nasal/conjunctival swabs were collected (prior to inoculation) day 0 to PID 10. Blood samples for complete blood count (CBC) were collected on day 0, and PID 3, 6, 8, and 10. Serum samples for serological studies were taken on day 0 and PID 3-10. Two lambs were selected randomly and killed on PID 5, 7, and 10. Tonsils, bronchial lymph nodes (BLN), mediastinal lymph nodes (MLN) , lung tissues from different lobes i.e., apical, intermediate and diaphragmatic, tissue portions from intestine i.e., duodenum, jejunum, ileum, colon and rectum, and from kidney were collected at the time of necropsy for viral isolation, fluorescent antibody study, and for histopathological examination. Bacteriological examination were also conducted on lung samples.

Cell Culture:

Ovine fetal corneal (OFC)[1] cells were grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100 units of penicillin and 100 ug of streptomycin sulfate per ml. Maintenance MEM was identical to growth medium except it contained 5% FBS. Cultures were planted in plastic screw-cap tubes, or 25 cm^2 flasks.

Virus:

Isolate 475N[2] was propagated in OFC cells in MEM maintenance medium. When 100% of cells were detached from the surface of the flasks due to cytopathic effects (CPE) of the virus, cultures were frozen and thawed three times and supernatant fluid was centrifuged at 500 x g for 20 minutes. The supernatant fluid was dispensed in 1-ml aliquots and stored at -20 C.

Virus Isolation:

Conjunctival/nasal (CN) swabs and fecal (F) samples were placed in tubes containing 2-ml of virus isolation diluent (VID). These swabs were pressed against the walls of tubes containing samples and removed. The VID was centrifuged 20 minutes at 500 x g and the supernatant fluid was then used to infect the OFC cell culture tubes.

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1 Received from Lemkhul National Animal Disease Center, Ames IA.

2 Recieved from Dr.Mattson, Veterinary Diagnostic Laboratory, College of Veterinary Medicine, OSU, Corvallis OR 97331.

Inoculated material was allowed to adsorb on to cell cultures for two hours in 37 C incubator with frequent rotations of inoculum. The inoculum was rinsed once and 1-ml of MEM (maintenance) containing gentamicin and fungizone was added. Cultures were then incubated at 37 C in an incubator and observed daily for CPE.

Tissue samples from lung (LNG), mediastinal lymph nodes (MLN), bronchial lymph nodes (BLN), tonsils, different portions of intestine and kidney were ground in a pestle and mortar into a 20% suspension. The samples were clarified by centrifugation at 500 x g for 20 minutes and supernatant was used to inoculate the OFC cell cultures tubes. Adsorption was carried out for two hours at 37 C with frequent rotations of cultures. Cell cultures were fed and incubated as per virus isolation from swabs. Inoculated tubes were then observed daily for CPE.

Inoculated cell culture tubes, both from CN, F and tissues showing CPE were freezed-thawed thrice. Samples which did not showed CPE were blind passaged three times on OFC cell culture. If no CPE was present after three passages, they were considered negative for the presence of virus. Virus pools were made from cultures showing CPE in 25 cm^2 flasks.

pH Sensitivity:

Different ovine and llama adenovirus isolates were tested for their sensitivity to acidic pH. Virus samples were diluted to 10^{-1} in Hank's medium adjusted to pH 3 (HCl) and the same sample was diluted in regular Hank's (pH 7.2); both samples were incubated at room temperature for 30 and 60 minutes. Samples were titrated in MEM medium to

10^{-8} and 0.1 ml was placed in each well of 96-well microtitre plate. Ovine fetal corneal (OFC) cells in MEM (growth) were added to each well. Plates were incubated in 2.5% CO_2 incubator at 37 C. Plates were observed for CPE on day 7.

Bacteriologic Examination:

Routine bacteriologic examinations were performed on lung samples for the presence of any secondary pathogen. Lung suspension (ground in physiological buffered saline) were inoculated on sheep blood agar plates.

Serology:

Virus neutralization tests were performed on all the viruses isolated in the study. Pools of isolated viruses were tested in 96-well microtitre plates. Isolated viruses were identified in a procedure identical to serum neutralization test (as described below) except the isolates were tested against hyperimmune serum prepared in rabbits to 475N. Virus neutralization (SN) tests were conducted on serums prepared from blood samples collected during the observation times. Serums were heat-inactivated at 56 C for 30 min and diluted in 2-fold steps using an initial serum dilution of 1:4. An equal volume of virus (50ul) which contained 100 $TCID_{50}$ was added to each serum dilution. The mixture was incubated 1 hour at 25 C at which time 50ul of growth medium containing $5x10^5$ OFC cells were added. One drop of sterile mineral oil was placed in each well and the plates were incubated at 37 C in an atmosphere of 2.5 percent CO_2. Cultures were examined for CPE 5 to 7 days later. Each serum was tested in duplicate and serum end-

point titres were defined as the last (most concentrated) dilution in the series which inhibited CPE in both dilution sets of serum. Viruses isolated from the lambs were confirmed as 475N if < or = 2 units of antibody neutralized 100 $TCID_{50}$ of isolated virus.

Fluorescent Antibody Technique (FAT):

Antiserum to 475N was prepared in rabbits. Culture flasks containing OFC cells were inoculated with the virus and, after all cells in the culture developed CPE, cells 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 x g for 20 minutes. The clarified virus suspension was subjected to isopycnic centrifugation (density 1.33g per ml) at 100,000 x g for 36 hours at 4 C. 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. Aliquot 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 -20 C 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 fluorescein isothiocyanate (FITC), was followed as previously described[3]. Sections of tissues were viewed with a Zeiss compound microscope using epifluorescence. Filtration of light was designed specifically for use with FITC. Sections of lung (from different lobes), bronchial and mediastinal lymph nodes, tonsils, intestine (from each segment), and kidney were prepared with a cryostat and tissue was placed on a glass slide. The tissue was dried at 37 C, then fixed in a 100 percent acetone solution for 20 min. After fixation, the tissue was again dried at 37 C for 20 minutes[4]. A few drops of diluted 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 37 C for 20 min 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 and negative control tissue was routinely prepared. Detection of viral antigen was apparent by the appearance of focal areas of intracellular fluorescence.

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[3] Dr.Mattson Veterinary Diagnostic Laboratory
College of Veterinary Medicine, OSU, Corvallis OR 97331.

[4] Idem foot # 4.

RESULTS

Clinical Response:

Lambs inoculated with isolate 475N developed mild clinical signs of depression, nasal, ocular discharge and loose feces. It was difficult to measure febrile response because of the relative warm environment and the physical effort the lambs made on being restrained. Lambs consistently showed a rectal febrile response ≥ 40 C beginning PID 3 (Table 2.1). As the infection progressed, especially (PID 4), lambs were less active, had a rapid respiration rate (≥ 24 per minute) and developed moderate cough. One of them (#968), showed severe signs of respiratory distress and was markedly depressed by PID 4. Lungs auscultation revealed mixed sounds ranging from muffled to respiratory rib friction beginning PID 4.

Necropsy Findings:

Lambs necropsied on PID 5 did not have any significant gross lesions in any tissues or organ except in the lungs (Fig 2.1, 2.2) and associated lymph nodes. In both lambs, irregular dark red areas of consolidation were found in the left anteroventral lung lobes which were clearly separated from normal-appearing lung tissue. Bronchial and mediastinal lymph nodes were moderately enlarged and hyperemic.

Lambs necropsied on PID 7 had almost identical gross pathologic findings as those of PID 5 (Fig 2.3, 2.4, 2.5), except that mesenteric lymph nodes were also enlarged. Pathology became more aggravated in lambs which were killed on PID 10 (Fig 2.6, 2.7,

2.8) in relation to pneumonia. Lung of both lambs showed marked collapsed of most of the right cardiac lobes, left cardiac and apical lobes of the lung.

Thoracic lymph nodes remained swollen and reddened and mesenteric lymph nodes were also found moderately enlarged. Beside these, no other gross lesions were observed in any other organ of the body.

Histopathology:

Histopathologic lesions were observed in lambs necropsied PID 5. Affected tissue of lung was completely consolidated and it was noted that no airways or alveoli were open (Fig 2.9). Bronchial epithelium appeared to be hypertrophic. Bronchial lumens were occluded with cell debris, mainly neutrophils, and sloughed epithelial cells. Sloughed epithelial cells contained large amphophilic intranuclear inclusion bodies (Fig 2.10) and lymphocytes were also observed in epithelium of bronchiole (Fig 2.12). Similar large inclusion bodies were scattered throughout the affected tissue (Fig 2.11) and were also seen in cells of alveolar and small bronchiolar origin. Inclusion bodies and areas of inflammation were seen in cells of the mucosa of nasopharynx.

Respiratory tract associated lymph nodes were hyperplastic and showed hemorrhage throughout the sinusoidal system with a few extraneous leukocytes. Mesenteric lymph nodes showed low levels of lymphoid hyperplasia. Section of intestine appeared normal. In some lambs, a moderate infestation of coccidia was detected on histopathology examination. On PID 5, no significant microscopic changes were observed in sections of the kidney, liver, spleen , gastrointestinal (GIT) and adrenal gland.

Lambs necropsied on PID 7 showed similar histopathology as those of PID 5. However, it was observed that there were fewer inclusion bodies in the bronchial epithelium and there was less cell debris occluding the bronchial airways. Tracheo-bronchial and mediastinal lymph nodes showed similar changes as seen in lambs necropsied on PID 5.

Pneumonic consolidation and lesions seemed to be more extensive in lambs necropsied on PID 10 but very few intranuclear inclusions were noted (Fig 2.13). It was also observed that some of the bronchioles within the affected area were clear (Fig 2.14). Bronchiolar epithelial hyperplasia and neutrophils were still active. Exudation was evident in airways and alveoli. Respiratory tract-associated lymph nodes were still hyperplastic but hemorrhage was not observed. Mesenteric lymph nodes appeared to be normal. On the basis of gross pathological and histopathological evidences, the lambs were diagnosed as having localized interstitial pneumonia.

Virus Isolation:

Viral isolates from the nasal/conjunctival, fecal and tissues of respiratory tract of lambs inoculated for the study were identified serologically as 475N. Virus was recovered from nasal/conjunctival secretions between PID 2 and PID 8, while in fecal samples, it was present in between PID 5 and PID 10 (Table 2.2). Gastrointestinal tract tissue samples and kidney tissue samples were negative for the presence of virus while lung tissue and associated lymphoid tissues were positive for the virus (Table 2.3). Virus was isolated from each lobe of lung and tonsils of both lambs necropsied on PID 5, but

it was recovered from the mediastinal lymph node of only # 968 lamb. Virus isolation was almost the same for the lambs necropsied on PID 7 as those of PID 5 (Table 2.3).

Virus was not isolated from any tissue of the lambs necropsied on PID 10 except lamb # 970 where only one lobe of the lung showed the presence of virus (Table 2.3). Most of the isolates showed the typical adenovirus CPE on their first passage on OFC cell cultures. Some cultures showed CPE beginning on the second passage while a few showed CPE on third passage.

pH Sensitivity:

A marked difference in pH sensitivity among different ovine adenovirus and llama adenoviruses was noted (Table 2.4). At least one log difference between samples incubated in Hank's pH 3 and Hank's regular medium (pH 7.2) was considered as pH sensitive. No titre difference among samples incubated at room temperature for 30 minutes and 60 minutes was noted. The strains of virus which were pH sensitive failed to produce any CPE after a 30 minute incubation.

Bacterial Isolation:

No significant bacterial isolates were found in any lamb necropsied on PID 5, 7, and 10 except from the lung of lamb # 970, in which a moderate growth of *Pasteurella hemolytica* was noted.

Serology:

Preinoculation serum prepared from all lambs showed no VN titre to 475N. Detectable antibody titers were first noted in sera of lambs on PID 6, and these titers increased progressively. A significant increase in titer was noted in serum prepared from blood samples on PID 9 and 10 (Table 2.5).

Immunofluorescence:

Tissues from the respiratory tract, gastrointestinal tract, lymphoid tissues and kidney of lambs necropsied on different PID's were stained and examined by fluorescence microscopy (Table 2.6). Gastrointestinal tract and tissues from kidney were negative in all lambs. All lobes of the lung of lambs necropsied on PID 5 were positive (Fig 2.15, 2.16) while mediastinal lymph node of # 969 and tonsil of # 968 showed the presence of the virus (Table 2.6). Viral antigen was detected in the apical lobe of both lambs (Fig 2.17) necropsied on PID 7 and virus was also demonstrated in the dia-phragmatic lobe of lamb # 971. Virus detection and intensity of fluorescence was very low in tissues examined from the lambs necropsied on PID 10, only diaphragmatic lobe of # 970 was positive for the virus (Fig 2.18 and Table 2.5).

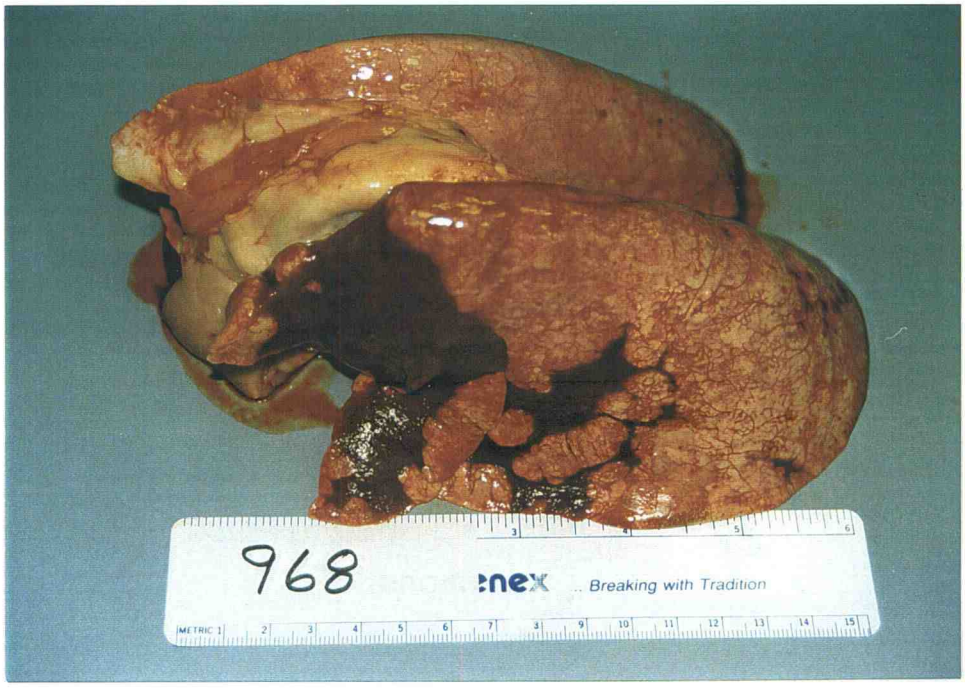

Figure 2.1. Lateral view of lung of a lamb No. 968 inoculated with ovine adenovirus isolate 475N, necropsied PID 5. Note dark red areas of collapsed lung.

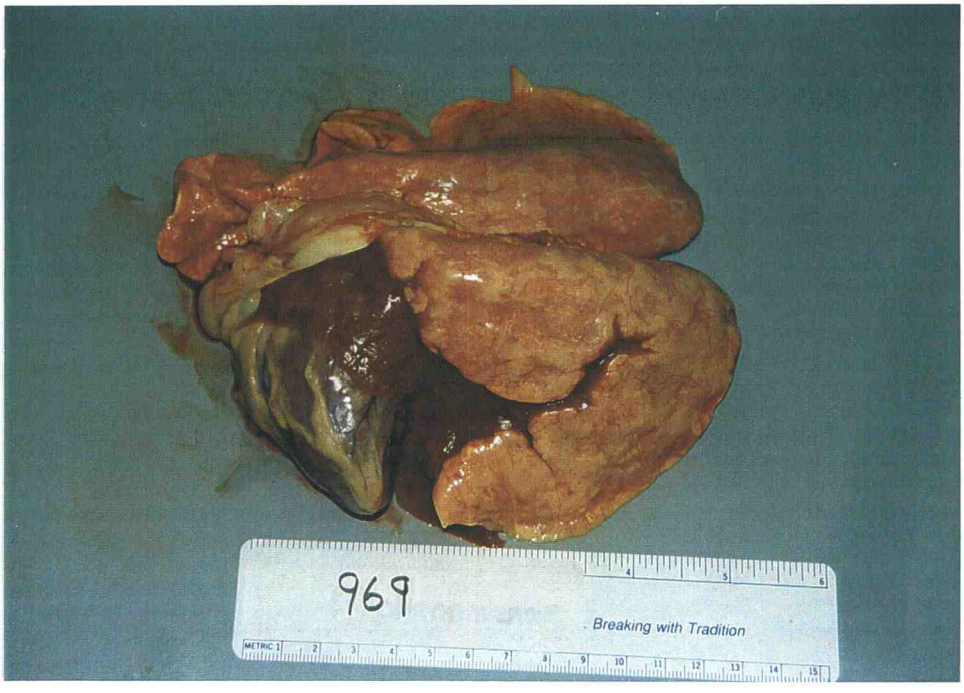

Figure 2.2. Lateral view of lung of a lamb No. 969 inoculated with ovine adenovirus isolate 475N, necropsied PID 5. Note dark red areas of collapsed lung.

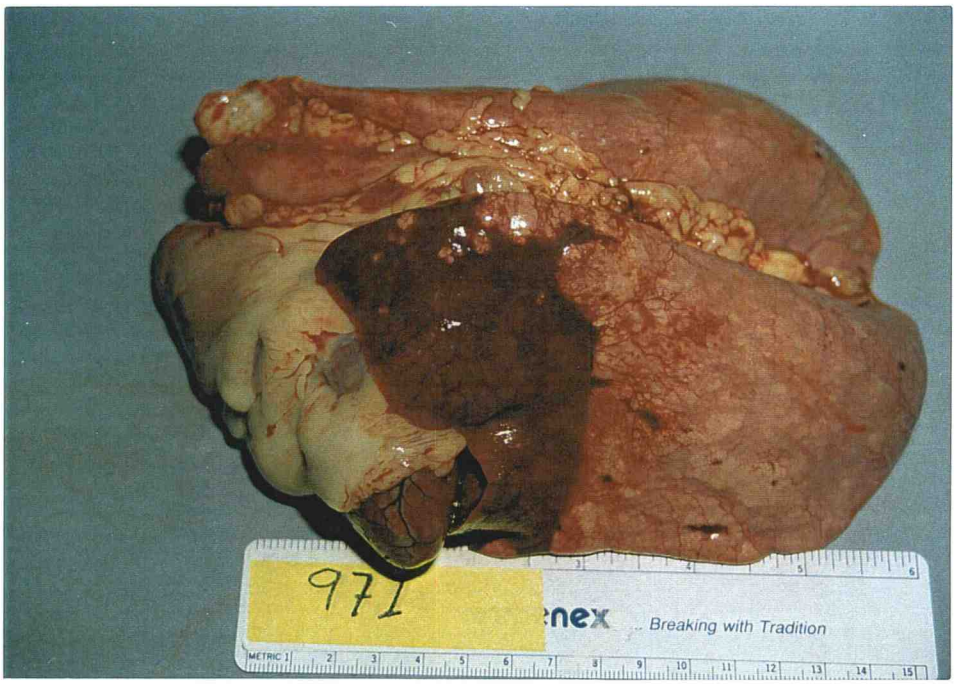

Figure 2.3. Lateral view of lung of a lamb No. 971 inoculated with ovine adenovirus isolate 475N, necropsied PID 7. Note dark red areas of collapsed lung.

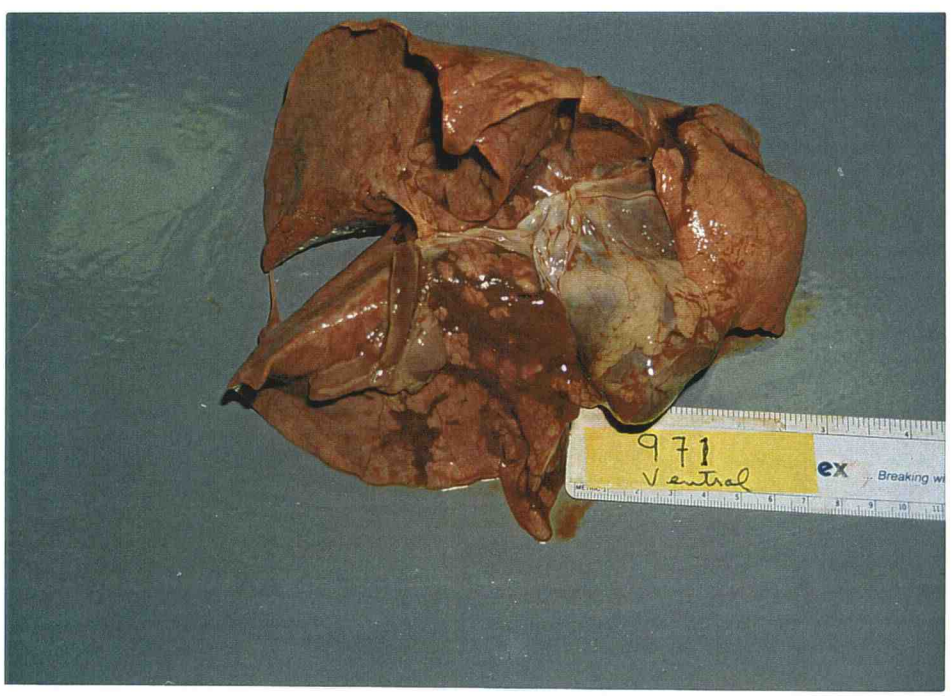

Figure 2.4. Ventral view of lung of a lamb No. 971 inoculated with ovine adenovirus isolate 475N, necropsied PID 7. Note dark red areas of collapsed lung.

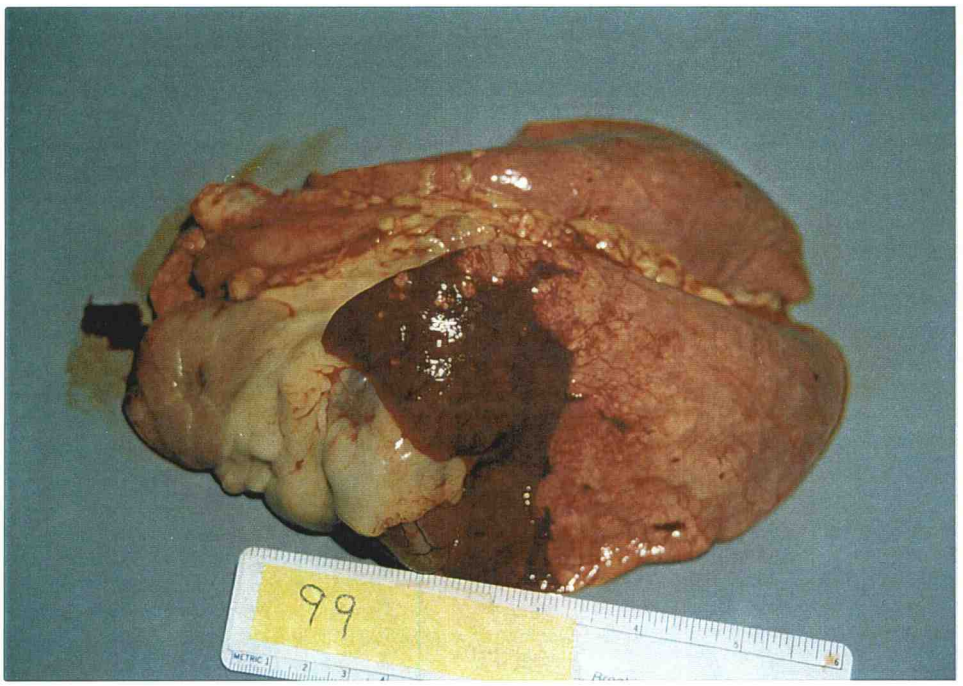

Figure 2.5. Lateral view of lung of a lamb No 99. inoculated with ovine adenovirus isolate 475N, necropsied PID 7. Note dark red areas of collapsed lung.

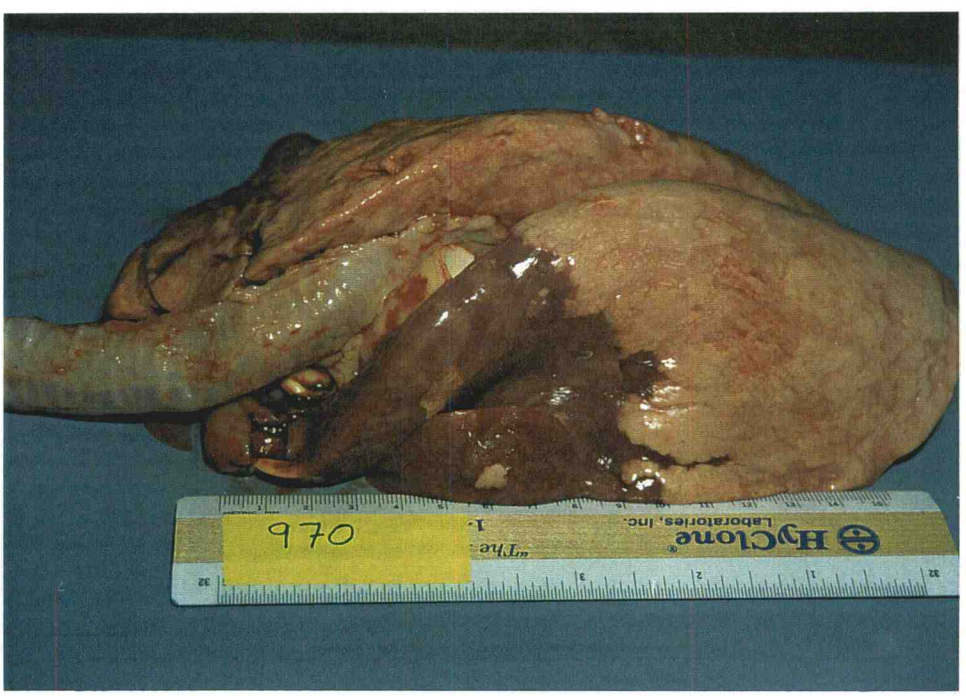

Figure 2.6. Lateral view of lung of a lamb No. 970 inoculated with ovine adenovirus isolate 475N, necropsied PID 10. Note dark red areas of collapsed lung.

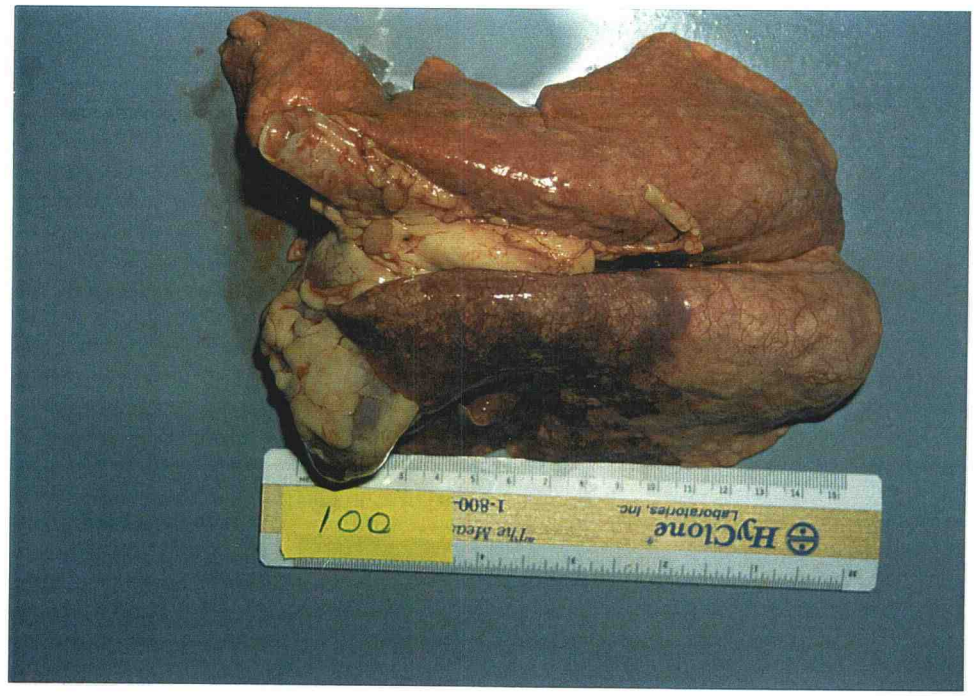

Figure 2.7. Lateral view of lung of a lamb No. 100 inoculated with ovine adenovirus isolate 475N, necropsied PID 10. Note dark red areas of collapsed lung.

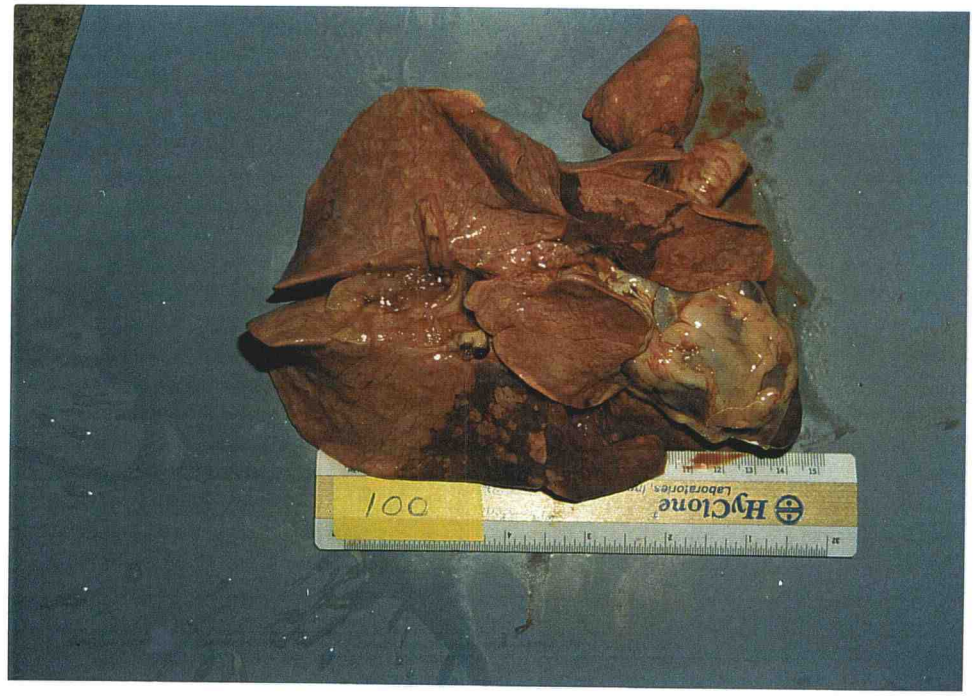

Figure 2.8. Ventral view of lung of a lamb No. 100 inoculated with ovine adenovirus isolate 475N, necropsied PID 10. Note dark red areas of collapsed lung.

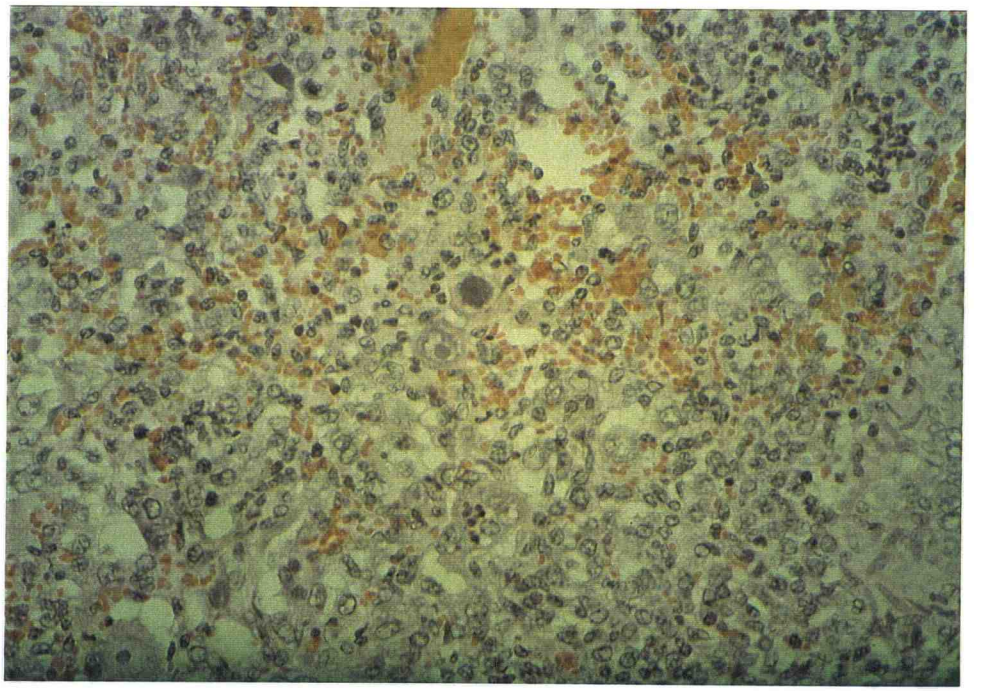

Figure 2.9. Photomicrograph of lung of lamb No. 968 inoculated with ovine adenovirus isolate 475N, PID 5. Note consolidation of lung with Excessive accumulation of exudative cells and large intranuclear inclusion body. H&E stain; X25.

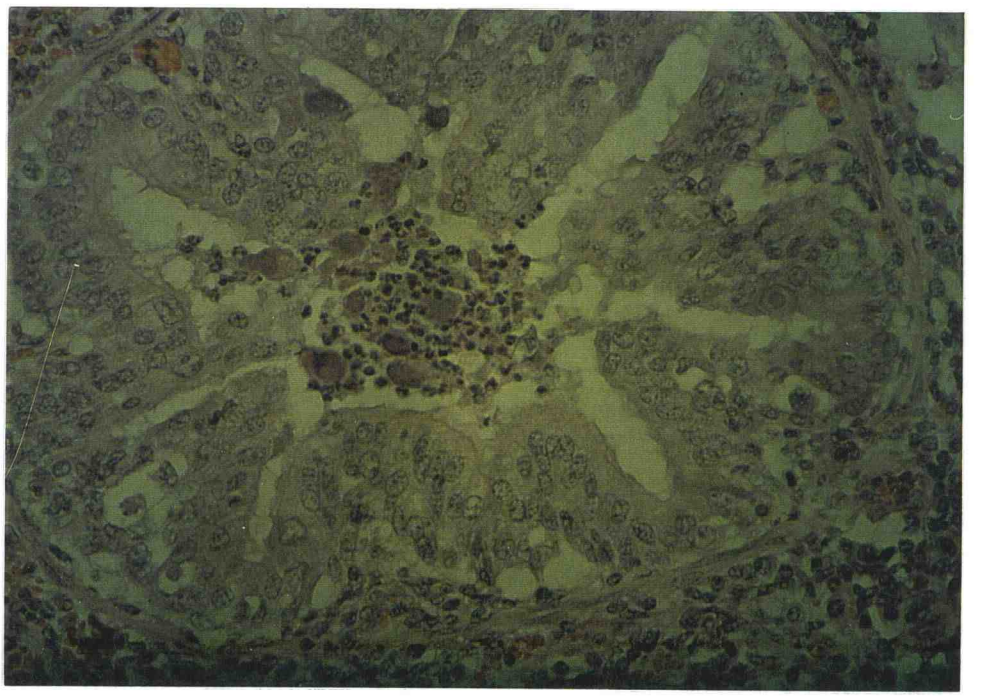

Figure 2.10. Photomicrograph of lung of lamb No. 968 inoculated with ovine adenovirus isolate 475N, PID 5. Note bronchiole with exudative cells having numerous large intranuclear inclusion bodies. H&E stain; X25.

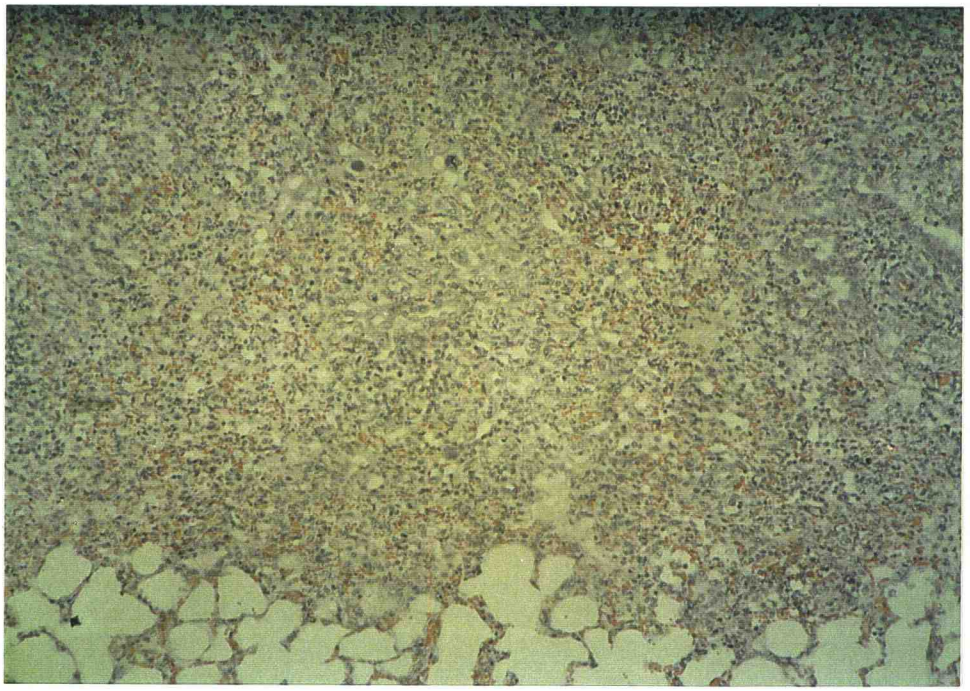

Figure 2.11. Photomicrograph of lung of lamb No. 969 inoculated with ovine adenovirus isolate 475N, PID 5. Note the clear interface between pneumonic lung with no open airways and normal lung with open airways, there are several inclusion bodies in cells. H&E stain; X25.

Figure 2.12. Photomicrograph of lung and large bronchiole of lamb No. 968 inoculated with ovine adenovirus isolate 475N, PID 5. Note the presence of lymphocytes in bronchiole. H&E stain; X10.

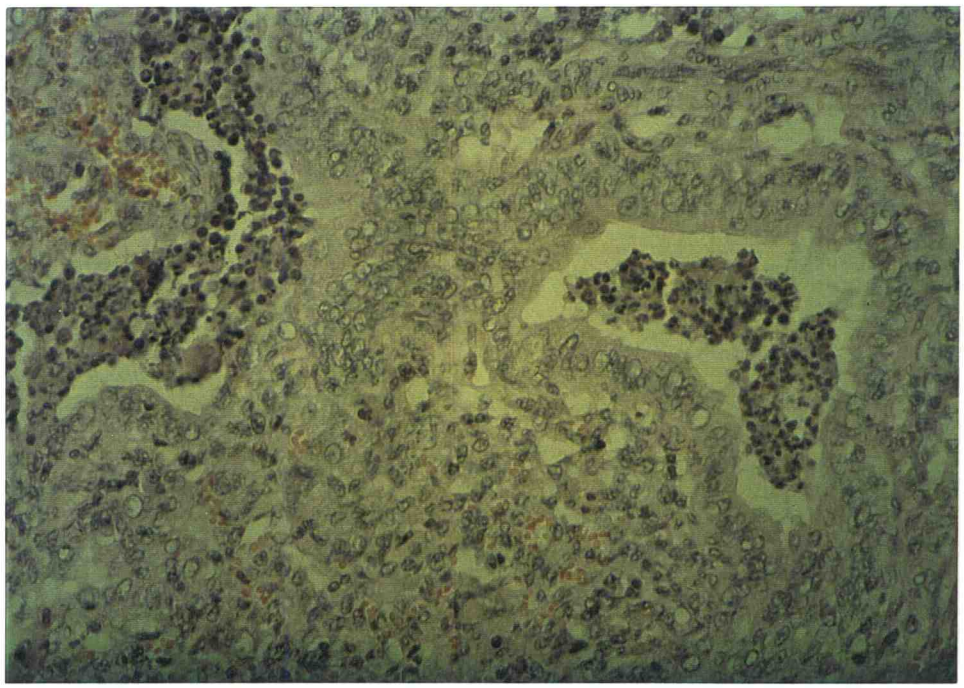

Figure 2.13. Photomicrograph of lung and large bronchiole of lamb No. 970 inoculated with ovine adenovirus isolate 475N, PID 10. Note that sloughed cells in the bronchioles do not contain inclusion bodies, indicating resolution of infection. H&E stain; X10.

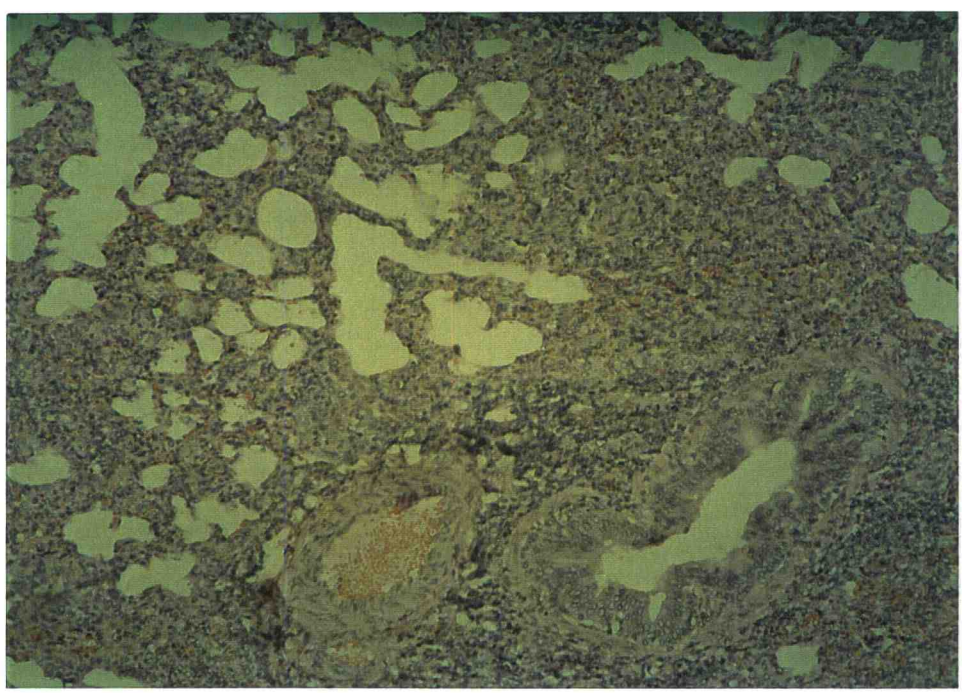

Figure 2.14. Photomicrograph of lung and large bronchiole of lamb No. 970 inoculated with ovine adenovirus isolate 475N, PID 10. Note the opening of bronchi and alveoli indicating resolution of infection. H&E stain; X10.

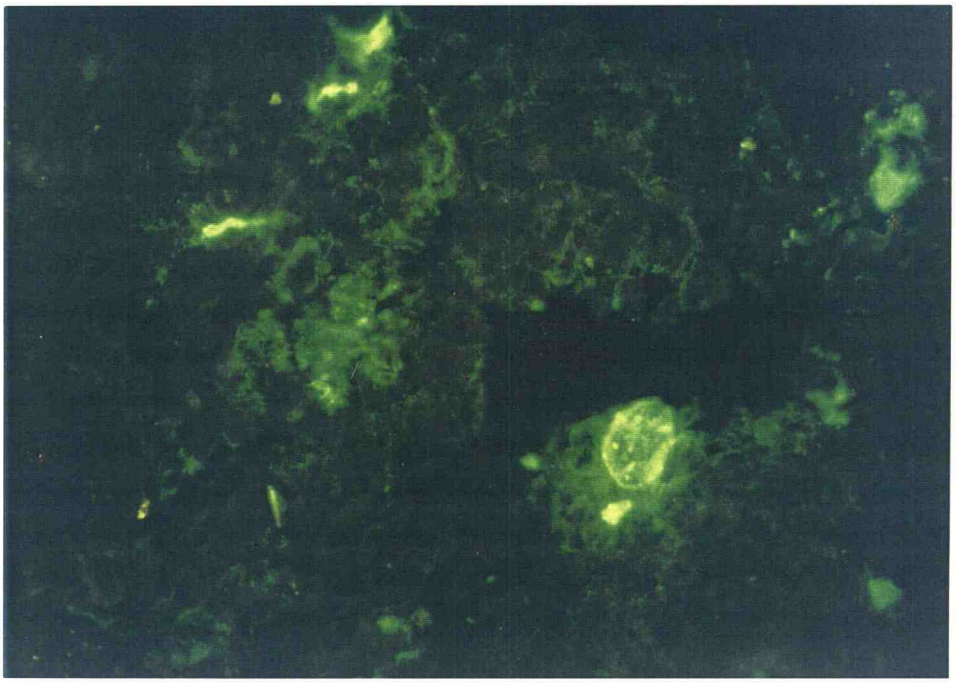

Figure 2.15. Photomicrograph of direct fluorescent antibody on lung of lamb No. 968 inoculated with ovine adenovirus isolate 475N, PID 5. Note presence of antigen in infected cells. FITC stain; X25.

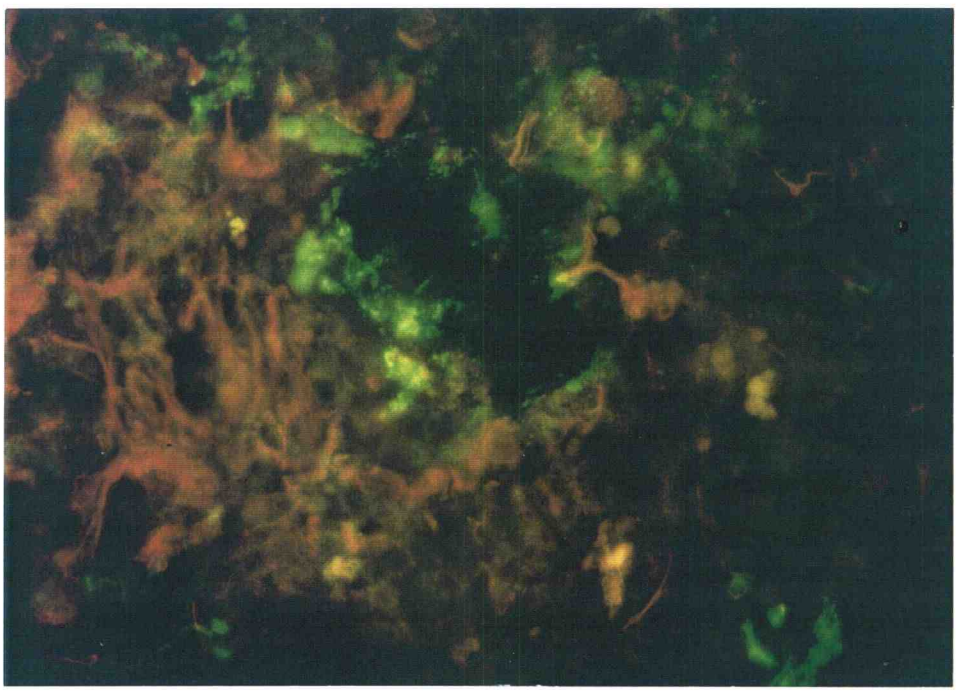

Figure 2.16. Photomicrograph of direct fluorescent antibody on lung of lamb No. 968 inoculated with ovine adenovirus isolate 475N, PID 5. Note presence of antigen in infected cells. Stained with FITC and counterstain with Evan's Blue; X100.

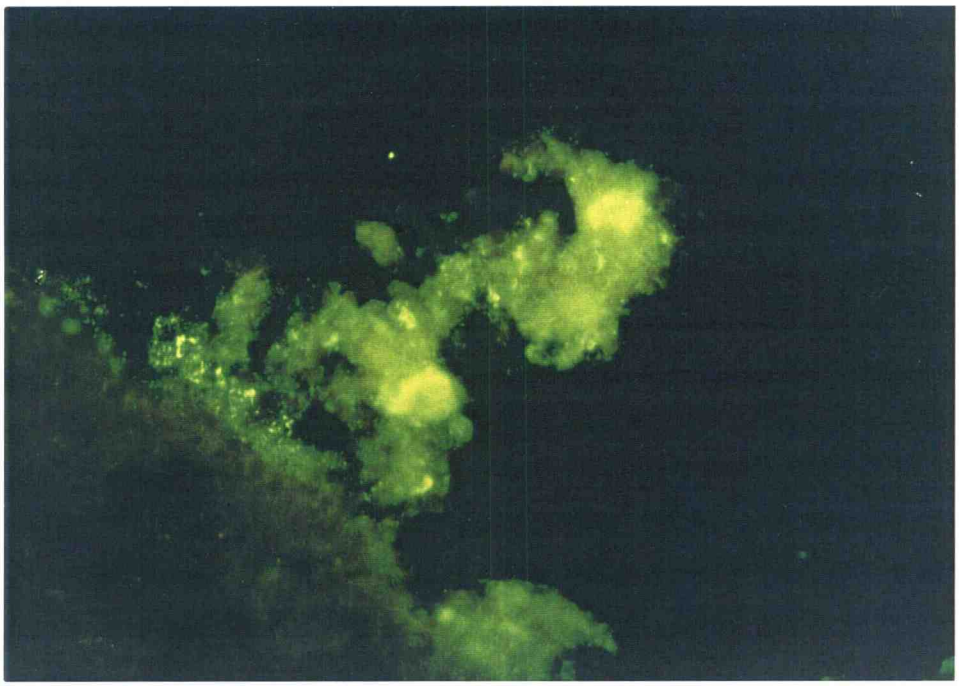

Figure 2.17. Photomicrograph of direct fluorescent antibody on lung of lamb No. 099 inoculated with ovine adenovirus isolate 475N, PID 7. Note presence of antigen in infected cells. FITC stain; X25.

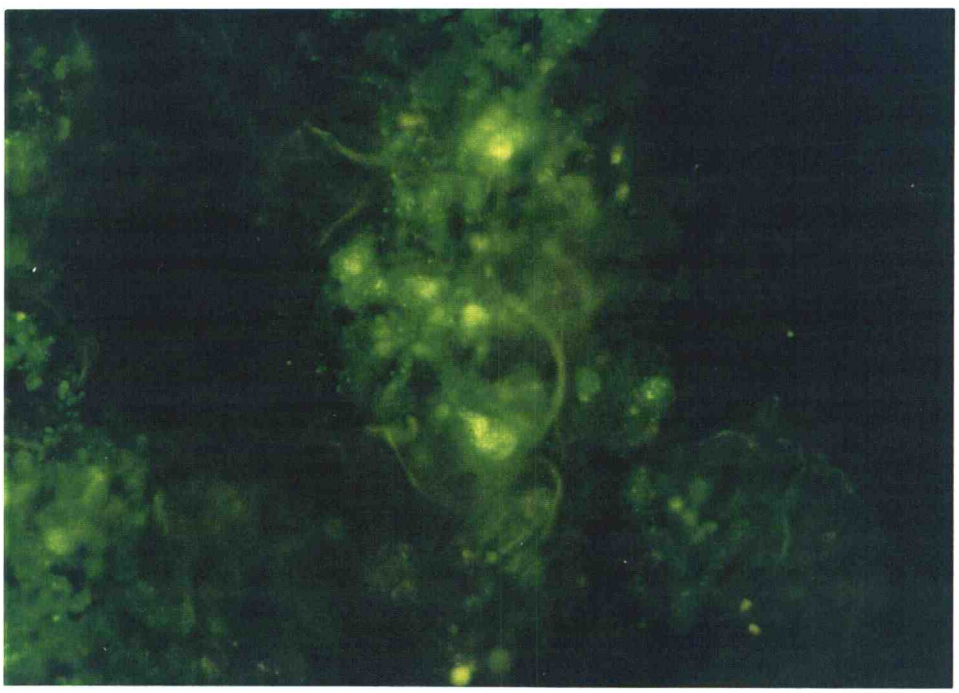

Figure 2.18. Photomicrograph of direct fluorescent antibody on lung of lamb No. 970 inoculated with ovine adenovirus isolate 475N, PID 10. Note presence of antigen in infected cells. FITC stain; X100.

Table 2.1. Rectal temperatures of lambs inoculated with ovine adenovirus isolate 475N on different days post-inoculation.

Temperature °C							
PID	#968	#969	#099	#971	#970	#100	Avgerage
0	40.0	38.8	39.5	39.8	40.5	40.0	39.8
1	40.5	39.4	39.7	39.7	39.6	39.7	39.8
2	39.9	39.8	39.7	39.7	40.2	40.1	39.9
3	40.7	40.7	40.3	41.4	40.0	40.0	40.5
4	40.7	40.7	41.0	40.5	40.2	40.5	40.6
5	41.2	40.2	40.3	41.6	40.7	41.0	40.8
6	NT	NT	40.7	41.3	41.2	41.7	41.2
7	NT	NT	39.5	40.8	40.0	41.7	40.7
8	NT	NT	NT	NT	40.2	40.2	40.2
9	NT	NT	NT	NT	40.0	40.4	40.2
10	NT	NT	NT	NT	39.5	40.5	40.0
Avg	39.9	40.4	40.6	40.1	40.3	40.5	40.3

NT= Not Tested.

Table 2.2. Virus isolation from nasal/conjunctival and fecal samples of lambs inoculated with ovine adenovirus isolate 475N on different days post-inoculation.

	968		969		099		971		970		100	
PID	CN	F	CN	F	CN	F	CN	F	CN	F	CN	F
0	-	-	-	-	-	-	-	-	-	-	-	-
1	-	-	-	-	-	-	-	-	-	-	-	-
2	+	-	+	-	+	-	+	-	+	-	+	-
3	+	-	+	-	+	-	+	-	+	-	+	-
4	+	-	+	-	+	-	+	-	+	-	+	-
5	+	-	+	-	+	-	+	+	+	-	+	+
6	N	N	N	N	+	-	+	-	+	-	+	+
7	N	N	N	N	+	-	+	-	+	+	+	+
8	N	N	N	N	N	N	N	N	-	+	+	+
9	N	N	N	N	N	N	N	N	-	+	-	+
10	N	N	N	N	N	N	N	N	-	+	-	+

N = Not tested (N).
- = No virus isolated
+ = Ovine adenovirus 475N isolated

Table 2.3. Virus isolation results from lambs at necropsy which were inoculated with ovine adenovirus isolate 475N.

Tissue	PID 5		PID 7		PID 10	
	#968	#969	#099	#971	#970	#100
Lung Api Lobe	+	+	+	+	-	-
Lung Acc Lobe	+	+	+	+	-	-
Lung Dia Lobe	+	+	-	+	+	-
Bron Lym Node	+	+	+	-	-	-
Medi Lym Node	+	-	-	-	-	-
Mese Lym Node	-	-	-	-	-	-
Duodenum	-	-	-	-	-	-
Ileum	-	-	-	-	-	-
Jejunum	-	-	-	-	-	-
Colon	-	-	-	-	-	-
Tonsil	+	+	+	+	-	-
Kidney	-	-	-	-	-	-

+ = Positive for 475N virus
- = Negative for 475N virus

Table 2.4. Results from pH sensitivity of different ovine adenovirus and llama adenovirus isolates.

		Titre[a]				
		30 minutes		60 minutes		
S#	Virus	pH 3	Control	pH 3	Control	Results
1	OAV-1	$3.98x10^6$	$1.99x10^6$	$3.16x10^6$	$1.26x10^6$	Resistant
2	RTS-151	-[b]	$8.51x10^3$	-	$1.20x10^4$	Sensitive
3	475-N	-	$3.98x10^3$	-	$3.16x10^3$	Sensitive
4	47-F	$1.38x10^5$	$2.29x10^5$	$1.58x10^5$	$1.58x10^5$	Resistant
5	OAV-6	-	$3.16x10^3$	-	$3.55x10^3$	Sensitive
6	RTS-42	$5.62x10^4$	$1.18x10^5$	$1.20x10^5$	$1.18x10^5$	Resistant
7	LA-7690	$1.00x10^4$	$1.99x10^4$	$1.20x10^4$	$5.01x10^4$	Resistant
8	LA-5330	-	$5.01x10^3$	-	$5.01x10^3$	Sensitive

[a] expressed as $TCID_{50}$/0.05 ml.
[b] - no CPE

Table 2.5. Serologic response of lambs inoculated with ovine adenovirus isolate 475N on different days post-inoculation.

PID	#968	#969	#099	#971	#970	#100
0	<1:4	<1:4	<1:4	<1:4	<1:4	<1:4
3	<1:4	<1:4	<1:4	<1:4	<1:4	<1:4
5	<1:4	<1:4	<1:4	<1:4	<1:4	<1:4
6	NT	NT	1:4	1:8	1:8	1:8
7	NT	NT	1:16	1:32	1:16	1:32
8	NT	NT	NT	NT	1:32	1:64
9	NT	NT	NT	NT	1:256	1:256
10	NT	NT	NT	NT	1:256	1:256

NT = Not tested.

Table 2.6. Virus detected by fluorescent antibody from lambs at necropsy which were inoculated with ovine adenovirus isolate 475N.

Tissue	PID 5		PID 7		PID 10	
	#968	#969	#099	#971	#970	#100
Lung Api Lobe	+	+	+	+	-	-
Lung Acc Lobe	+	+	-	-	-	-
Lung Dia Lobe	+	+	-	+	+	-
Bron Lym Node	+	-	-	-	-	-
Medi Lym Node	-	+	-	-	-	-
Mese Lym Node	-	-	-	-	-	-
Duodenum	-	-	-	-	-	-
Ileum	-	-	-	-	-	-
Jejunum	-	-	-	-	-	-
Colon	-	-	-	-	-	-
Tonsil	+	-	-	-	-	-
Kidney	-	-	-	-	-	-

+ = Positive for virus detection
- = Negative for virus detection

DISCUSSION

A frequently used procedure to determine if a specific virus could potentially induce disease in young lambs is to experimentally produce infection in the subjects and determine the severity of disease. Accordingly, the goals of this project were to experimentally inoculate six young lambs with isolate 475N and observe clinical signs, measure degree of pathological changes, virus shedding patterns and the dynamics of the humoral immune response.

Lambs inoculated with isolate 475N showed a mild clinical response and moderate pathology with gross and microscopic pathologic changes limited to the respiratory tract and tonsils. Considering the fact that the virus was detected by virus isolation and FAT from tissues of the respiratory tract and there was minimal or no pathologic changes or virus detection in the intestinal tract, it was concluded that isolate 475N is a potential respiratory tract pathogen.

Interestingly, when acid sensitivities of different OAV isolates were examined, it was observed that some isolates were labile to low pH while some were stable; isolate 475N is labile to pH 3.0. In two different studies with isolate RTS-151 and RTS-42 (related to OAV-6 and OAV-5, respectively), it was reported that isolate RTS-151 was acid (pH 3) labile[45] while RTS-42 was acid resistant[42]. In the original studies of the prototypes of OAV 1-5, the viruses were reported to be acid resistant.[7, 50] In the first report and description of OAV-6, acid sensitivity was not determined.[20] However, in the current study, it was found that this prototype virus is acid labile. It appears that isolate 475N and RTS-151, both of which are antigenically related to OAV-6, follows the same

pattern. In another study with ovine adenoviruses which were related antigenically to OAV-6, all of the isolates were found to be acid labile.[55]

It appears that some difference in adaptation of OAV strains exits. Those isolates which were recovered from respiratory tract tissue with only respiratory tract disease signs are acid labile while those isolated from intestine and respiratory tract tissues and which induce pneumoenteritis are acid resistant. This observation appears to be consistent with the two different LAV isolates which were tested for this property (Dr.Mattson unpublished data).[46] Llama adenovirus isolate (94:5330), which was recovered from the nasal cavity, is acid labile while the other (isolate 7649), which was isolated from the feces of a cria, is acid resistant. The question that arises, is this property an important criterion to be considered for the establishment of infection and can we group adenoviruses based on this property like we have with apthovirus, rhinovirus, and enterovirus of *Picornaviridae*?.[32] After reviewing the literature, it is felt that most investigators working with adenoviruses have not examined this property and even the International Committee on Adenoviridae did not recognize its significance.[69] It appears that adenoviruses that infect the intestinal tract of the neonates should be able to withstand acid conditions to a pH 3.0 (abomasum), as the rumen is not fully developed and is also bypassed by the reticular groove. Newborn ruminants basically function as a monogastric animal and maintain a low gastric pH.[40, 57, 68]

When lambs were first necropsied on PID 5, it was noted that virus could be detected readily by virus isolation and fluorescent antibody examination. By PID 10, virus could be detected with difficulty by fluorescent antibody technique only. Similarly,

the pathology in respiratory tract tissues (particularly in lungs) was more severe in animals necropsied in the early phase of infection than those which were necropsied on PID 7 and PID 10. This apparent elimination of free virus and virus-infected cells corresponded with the production of antibodies.[21, 22, 42, 44, 45] Although parameters of the cell-mediated immune response were not measured, however,it is assumed this also is an important mechanism whereby the animal overcomes infection with these intracellular pathogens.[32, 65]

It is noted that studies involving experimental infection of animals with viruses usually are designed to determine the pathogenesis of infection. Rarely can it provide a true understanding of the role of the agent in the corresponding disease syndrome. For example, several studies have shown that lambs experimentally infected with OAV or Pasteurella species alone, resulted in low mortality and minimal pathologic changes. However, when viral infection was followed by challenge with Pasteurella species, pathologic changes and morbidity was much higher.[21, 44] It is noted in these studies that presence of VN antibodies had little effect on the course of the disease after virus infection was established. Initial virus infection was believed to allowed bacteria to colonize and produce toxins which resulted in extensive pathology. In the current study, lambs were held in strict isolation. If they had been housed under natural conditions or exposed to a variety of microorganisms which could have serve as secondary bacterial invaders, clinical signs of disease and pathologic changes could have been more severe.

In preliminary studies which were conducted when 475N was first isolated, the virus produced clinical signs of anorexia, cough, elevated rectal temperatures, and

respiratory distress when inoculated in young lambs.[67] When the subjects were necropsied on PID 3, it was noted that large portions of lungs were swollen, dark red, and firm. Histopathological studies revealed the presence of large, basophilic intranuclear inclusion bodies in lungs as well as sloughed epithelial cells in the bronchi. It was also noted that some bronchioles were filled with neutrophils which assisted in producing consolidation of lung parenchyma. Virus was isolated from nasal/ conjunctival, fecal swabs, and tissues of respiratory tract while no virus was detected in kidney, spleen, liver, and tissue samples from gastrointestinal tract.[67] Results of the present study were consistent with this preliminary study. Virus isolation from the feces can either be explained by minimal replication and virus shedding from the intestinal tract or from shedding of the virus from the tonsil and respiratory tract into the feces.[45] In a similar study using OAV strain RTS-151 (antigenically related to 475N), 4-weeks old colostrum-deprived lambs were inoculated intratracheally. Virus was recovered and produced similar mild clinical signs including severe respiratory tract pathology.[45] Virus was isolated from nasal/conjunctival samples, tracheal fluid, and from explants of lungs. Virus was not recovered from any other organ including the gastrointestinal and urinary tract.[45] On the basis of pathology and other clinical observations, it was suggested that this strain, later shown to be related to OAV-6, should be considered potentially pathogenic to young lambs. There is a close similarity between the results obtained from RTS-151 and 475N in the current study. The same conclusion can be formulated with isolate 475N in that it is a potential pathogen for the respiratory tract of lambs.

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CHAPTER III

DNA Restriction Analysis of Ovine Adenoviruses 1 to 6 and Selected Unknown Strains of Bovine, Ovine and Llama Adenoviruses.

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ABSTRACT

Eleven adenovirus strains including six ovine prototypes, three uncharacterized ovine and bovine isolates and two uncharacterized llama isolates were digested with four different restriction enzymes (RE). This investigation was conducted in order to identify their specific RE patterns which may help in comparing different isolates and assist in their classification. Digested viral and cellular DNA samples were separated in 0.7% agarose gels, stained with ethidium bromide and visualized using an ultra violet light source. The enzymes *Bam* HI, *Eco* RI, *Hind* III, and *Pst* I were sufficient for differentiating the strains. Each enzyme digested the DNA of the viral sample differently and produced varying number of RE bands, ranging from 2-10. Digestion with the *Pst* I enzyme produced more RE bands than others, while *Bam* HI yielded less bands. Enzyme *Pst* I cleaved all the viral strains (13/13) into different number and size of bands while *Eco* RI did not digest all the strains (10/13). These four enzymes also cleaved both llama isolates and produced different RE patterens. Several of the viral isolates which were classified antigenically within a species yielded RE bands which were not identical by their RE patterns.

INTRODUCTION

Prior to the late 1970's, antigenic differences within various groups of viruses were defined by serological procedures. But, with the development of molecular techniques including isolation of viral nucleic acid from infected cells, a number of

procedures were used to demonstrate minor difference in the genome of virus strains within the same virus group. Included in these many molecular techniques, is the cleaving of the viral genome with different restriction endonucleases (RE) and separating the cleaved bands by electrophoresis (commonly known as DNA fingerprinting). This procedure is now frequently used for differentiating and classifying various strains of viruses. This technique is relatively easy to adopt and results are often used as the final assay of distinguishing virus strains.

There are six serologically defined ovine adenovirus strains but there are no reports of their DNA fingerprinting pattern. There is only one report that described the RE patterns of US ovine adenovirus isolates RTS-42 and RTS-151. This report did not mentioned the number or size of RE bands produced by different restriction enzymes.[23] There are several reports regarding this technique in the literature for human[1], bovine[3], and porcine[18] adenoviruses. The current study was conducted to describe the RE patterns of the six recognized ovine adenovirus and to compare them to some ovine, bovine and llama adenovirus isolates which have not been classified.

LITERATURE REVIEW

Adenoviral Classification:

Rowe and coworkers first isolated a cytopathogenic agent from human adenoids and proposed the term Adenoid Degeneration Agent in 1953.[24] Hilleman and Werner isolated these viruses from patients with acute respiratory illness and used the term

Respiratory Illness Agent.[14] Huebner and colleagues in 1954, proposed the term Adeno Pharyngeal Conjunctival agents as the group name.[17] In 1956, Enders proposed adenoviruses as the group name for these viruses.[5] Lastly, the International Committee on the Taxonomy of Viruses, a virology section of International Association of Microbiological Societies, established the formerly genus adenovirus into the family *Adenoviridae*. This family is represented by two genera which consists of Mastadenovirus (adenovirus of mammals) and Aviadenovirus (adenovirus of avians).[7]

Characteristics of Adenoviruses:

The characteristics of adenovirus have been defined by Ginsberg,[10] Huebner *et al.*,[17] and Willner.[28] Adenoviruses from all species are nonenveloped, icosahedral, 700-800 **Å** in diameter, of a density of 1.34 g/ml in CsCl, and of a particle weight of about 175 x 10^6 daltons. All adenovirus contain linear double-stranded deoxyribonucleic acid (DNA) of 29 x 10^6 daltons molecular weight (30-37 kilobase pair).[9] Associated with their genome there is a 55K protein, which plays a role in replication[8] and also gives a unique advantage in separation of viral genome from the fragmented cellular DNA of infected cell.[25] The icosahedral protein shell (capsid) has a 5:3:2 cubic symmetry pattern and consists of 240 hexons (each bound to six neighbors) and, at the apices, 12 pentons (each bound to 5 neighbors) which carry fibers; a fiber on each penton.[11] The hexons are polygonal prisms with a central hole.[26] The pentons, which are more complex, consist of similar polygonal base with a fiber attached. The fibers vary in length (100 - 310 Å) and are 20 Å in diameter. The fibers have a terminal knob measuring 40 Å in diameter.[20]

Adenoviruses are resistant to the action of lipid solvents, such as ether,[17] chloroform,[6] fluorocarbons,[27] and deoxycholate[16] indicating that the viruses lack essential lipid. Adenoviruses are acid stable[13] (with few exception i,e., few strains are sensitive to pH 3) and, in fact, are more stable at acid than alkaline pH.[27] They are also relatively stable in homogenates of infected cells.[4]

Purification of Adenovirus DNA:

Several different procedures are described in the literature for the isolation of viral DNA from infected cells. Originally Green et al., described the technique to purify adenoviral DNA from KB cells monolayers.[12] Subsequently, additional modifications in the original procedures were made.[15, 21] Presently two procedures are common for the purification of viral DNA from infected cells. The first procedure is to simply cultivate the virus in appropriate cell cultures and then lyse the infected cells with sodium dodecyl sulfate (SDS), phenol extract the proteins, and precipitated the viral DNA. The other procedure involves virus purification by ultracentrifugation (CsCl density gradient), phenol extraction of proteins and viral DNA precipitation. Each procedure has its own advantages and disadvantages. The later described procedure is not as convenient for the purification of viral DNA from many different samples or for small scale preparation of non-labeled DNA[25] as it requires the additional work of labelling and ultra-centrifugation.

There are two types of nucleases with specific biological properties, exonucleases and endonucleases. Exonucleases charasterically cleave DNA at 5′ and 3′ end while

endonuclease are those that catalyzes cleavages within a nucleic acid strand. Among endonucleases there is a special class of enzymes, restriction enzymes (RE), that recognizes a specific nucleotide basepair sequance and cleaves the DNA strand at these sites. For example, restriction enzyme *Eco* RI recognize hexanucleotide sequence of 5′ G↓AATTC and 3′ CTTAA↓G i.e., wherever this sequence will appear in the strand of DNA, it will cleave at this site and will produce different number of bands.[19] The following lists six basepair the endonucleases used in this study and the specific sites where the nucleic acid is cleaved.

Specifities of restriction enzymes[a].

Enzyme	Bacterial Source	Restriction Site.
Bam **HI**	*Bacillus amyloliquefaciens* **H**	G↓GATCC
Eco **RI**	*Esherechia coli* **R245**	G↓AATTC
Hind **III**	*Haemophilus haemolyticus*	A↓AGCTT
Pst **I**	*Providentia stuartii*	CTGCA↓G

[a] adopted from Biochemistry by Mathews CK and van Holde KE 1990, p 866.

MATERIALS AND METHODS

Species of Ovine Adenovirus:

Six prototypes of ovine adenovirus(OAV)[5] and two US OAV (RTS-42 and RTS-151) three local ovine, bovine and two local isolates of llama adenovirus(LAV) were used for the study. Isolates 475N and 47F were isolated from two different lambs with respiratory and pneumoenteric problems, respectively.[29] Isolate 32CN was isolated from a bovine with conjunctivitis and respiratory disease.[29] Llama adenovirus isolate 7649 was isolated from feces of a cria while isolate LA 5330 was recovered from the nasal cavity of a llama at necropsy (Mattson Unpublished data).

Cell Cultures:

Ovine fetal corneal cells (OFC) and llama kidney cells (LMK-1) were grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100 units of penicillin and 100 μg of streptomycin sulfate per ml. Maintenance MEM was identical to growth medium except it contained 5% FBS.

Growth of Ovine and Llama Adenoviruses:

All strains of ovine, bovine and llama adenoviruses were grown either in ovine fetal cornea (OFC) or llama kidney (LMK-1) cells, respectively. Prior to inoculation,

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

culture flasks (25 cm^2) were rinsed with PBS thrice in order to remove old culture media. Viruses were inoculated (0.5 ml per flask), containing 10^5 $TCID_{50}$/ml of virus and then placed on a rocker platform for adsorption in an incubator maintained at 37 C for two hours. After adsorption, inoculum was decanted and flasks were fed with fresh MEM supplemented with 5% FBS and incubated at 37 C. Flasks were observed daily for CPE, which was usually evident between 4-5 days post-inoculation. When more than 90% cells were affected, media was removed and flasks were rinsed three times with PBS (pH 7.4).

Separation of Adenovirus DNA from OFC and LMK-1 Cellular DNA:

Cells were lysed by adding 0.6% SDS, 10 mM EDTA and 275 μg/ml Pronase. Treated flasks were then incubated at 37 C for one hour on a rocking platform. Cells were scraped with a plastic scraper and collected in 1.5 ml eppendrof tubes. Viral DNA was partially purified by adding a 5 M solution of sodium chloride (NaCl) to the DNA solution (final concentration of NaCl was 1 M). Samples were allowed to set overnight at 4 C. This step precipitated much of the high molecular weight cellular DNA. Uninfected OFC and LMK-1 cells were also processed in the same fashion with the exception of addition of NaCl.

Extraction of Adenovirus DNA and Cellular DNA:

The samples were then microcentrifuged for 30 sec and supernatant was removed carefully leaving most of the cellular DNA in the bottom of the microfuge tube.

Supernatant fluid containing predominantly viral DNA was transferred to a 1.5 ml microcentrifuge tube. Equal volume of phenol:chloroform:isoamyl alcohol (P:C:IAA) (25:24:1) was added to the DNA solution and vortexed briefly, and samples were microcentrifuged for 30 sec at full speed (12,400 X g). Upper layer (viral DNA) was carefully separated from the bottom P:C:IAA without disturbing the interphase. This step was repeated twice, as this step removed degraded proteins from the DNA solution. The viral DNA was precipitated by adding two volumes of cold (-20 C) absolute ethanol to each sample and cooling to -70 C and holding 4-18 hrs. After control cells were lysed by SDS-Pronase, the cellular DNA was precipitated by adding 2.5 M sodium acetate at a final concentration of 0.25 M and then by adding cold (-20 C) absolute ethanol and cooling to -70 C, like viral DNA samples.

Cellular or viral DNA was collected by microcentrifugation for 30 minutes at 4 C at full speed (12,400 X g). In most of the samples, the DNA pellet was adherent to the walls of microcentrifuge tubes. Ethanol was decanted and the DNA pellet was washed twice with 70% ethanol (room temperature). After final washing and discarding of ethanol, tubes were dried in a hood for 30 minutes. The DNA pellet was resuspended in 30 µl of TRIS-HCl (pH 8.1). Then samples were placed on a hot plate (65 C) for about 10 minutes and shaken for one hour at 37 C in order to dissolve the DNA pellet. The DNA samples were then quantified by using a spectrophotometer (260 nm wavelength).

Restriction Enzyme Digestion of DNA Samples:

Four different restriction enzymes *Bam* HI, *Pst* I, *Hind* III and *Eco* RI were used to digest the viral and cellular DNA. Using buffers, specific to each RE (provided by the supplier), digestion was carried out in a 20 µl volume (quantity of DNA in each sample was higher than 5 µg) , containing Ribonuclease A (1 µl of 100 µg/ml). Digested samples were incubated at 37 C for 90 minutes. The digestion process was stopped by adding 5 µl loading buffer, containing EDTA (as chelator), to each sample. Following digestion, samples were loaded on 0.7% agarose gels.

Electrophoresis:

Bands produced by RE were separated by electrophoresis in a horizontal agarose gel electrophoresis apparatus. The digested viral and cellular DNA samples were loaded into agarose gel wells in Tris acetate buffer and a constant 80 Volt current was supplied for approximately 3.5 hours. Gels were stained in a solution of ethidium bromide (0.1µg/ml) at 4 C. Restriction bands were visualized by using an ultraviolet light (300 nm) transilluminator and photographed with a polaroid camera on a polaroid type film (#667 8.5x10.8cm). Photographed gels were sccaned in order to transfer their images on to a papper. Standard molecular weight markers were used to determine range of sizes of RE fragments. Bands showing under 0.8 Kbp across molecular weight markers were not considered in totalling.

RESULTS

Purification of Adenovirus DNA:

The procedure adopted to purify viral DNA from the infected OFC and LMK-1 cells consistently left some traces of cellular DNA in all samples prepared. This appeared as a white streak in each lane background when separated on the agarose gel. Concentration of viral DNA usually was ≥ 0.45 μg/μl for each DNA sample while A_{260}/A_{280} ratio was ≥ 1.7 for most of the samples.

Restriction Enzyme Digestion of DNA Samples:

All viral DNA samples produced banding patterns when digested with the four different restriction enzymes (Table 3.1). All four RE's digested both llama adenovirus isolates (Fig 3.1) and yielded different numbers and sizes of RE bands. Restriction enzyme *Pst* I produced more fragments and it cleaved all the adenoviral DNA samples 13/13 (Table 3.1, Fig 3.2), while *Bam* HI cut 12/13 samples (Table 3.1, Fig 3.4), RE *Hind* III cleaved 10/13 (Table 3.1, Fig 3.5) and *Eco* RI digested also digested same number of samples 10/13 (Table 3.1, Fig 3.3).

Among the RE used, enzyme *Pst* I appeared to be most useful as it cleaved all adenovirus strains (13/13, Table 3.1). The number of bands produced by this RE ranged between 5-10 (Table 3.1), i,e., there were four to nine cleavage sites in all adenoviruses as recognized by this RE. Restriction enzyme *Pst* I did not produced any visible band when used to digest DNA of uninfected OFC (Fig 3.2) and LMK-1 cells (Fig 3.1).

Restriction enzyme *Bam* HI digested all (12/13) adenovirus strains except isolate 47F (Table 3.4). The number of bands generated by this enzyme ranged between 2-6 i,e.,

there are only one to five restriction sites. Uninfected OFC (Fig 3.3) and LMK-1 (Fig 3.1) did not produced any visible bands when digested by this RE.

Although RE *Hind* III did not cut all adenovirus strains (10/13), it produced more bands than *Bam* HI and *Eco* RI (Table 3.1). The number of bands produced by this RE ranged between 3-10 i,e., having two to nine cleavage sites. Restriction enzyme *Hind* III did not produced any visible band when used to digest DNA of uninfected OFC (Fig 3.4) and LMK-1 cells (Fig 3.1).

Restriction enzyme *Eco* RI appeared to be similar as *Hind* III in digesting all adenovirus strains (10/13) than others (Table 3.1). It yielded 2-6 bands i,e., have only one to five restriction sites. This RE did not produced any visible band when used to digest DNA of uninfected OFC (Fig 3.5) and LMK-1 cells (Fig 3.1).

Table 3.1. Number of bands of different ovine and llama adenoviruses after digesting with four different restriction enzymes.

S#	Sample Type	.....# of Bands.....			
		Bam HI	*Hind* III	*Pst* I	*Eco* RI
01	OAV-1	5	9	8	4
02	OAV-2	2	7	4	4
03	OAV-3	2	8	6	4
04	OAV-4	2	9	6	4
05	OAV-5	4	9	7	2
06	RTS-42	4	10	8	3
07	OAV-6	4	UC	9	UC
08	RTS-151	4	3	8	UC
09	475N	6	UC	7	2
10	32CN	5	UC	7	UC
11	47F	UC	8	8	3
12	LA7649	2	5	5	7
13	LA5330	2	4	5	5
14	OFC	-	-	-	-
15	LMK-1	-	-	-	-
16	MM-9780	23.13	9.41	6.55	4.36
17	MM-0672	01.35	1.07	0.60	0.31

Legends.

OAV	Ovine adenovirus	LA	Llama adenovirus
M Wt	Molecular Weight	Kbp	Kilobase pair
UC	Uncut	OFC	Ovine fetal corneal cells
LMK-1	Llama kidney cells	MM	Molecular Markers
-	No bands or not estimated	B #	Number of Bands

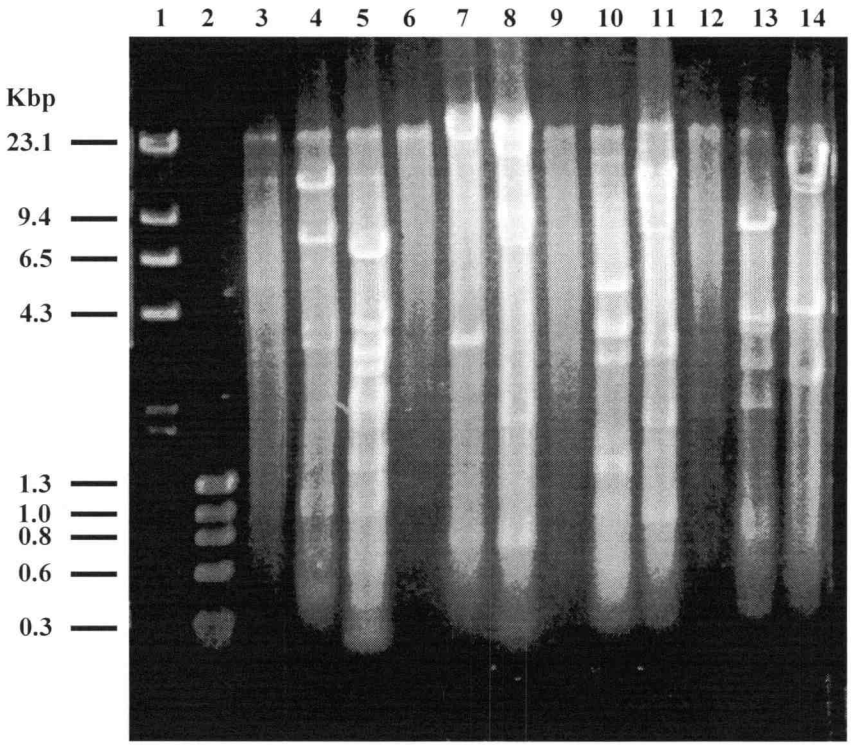

Figure 3.1. Restriction patterns of Llama adenovirus (LAV) isolates DNA as digested with *Pst* I (Lane 3,4,5), *Bam* HI (Lane 6,7,8), *Hind* III (Lane 9,10,11) and *Eco* RI (Lane 12,13,14).

Lane # 1.	MW Marker	Lane # 8.	LAV 5330
Lane # 2.	MW Marker	Lane # 9.	LMK-1
Lane # 3.	LMK-1	Lane # 10.	LAV 7649
Lane # 4.	LAV 7649	Lane # 11.	LAV 5330
Lane # 5.	LAV 5330	Lane # 12.	LMK-1
Lane # 6.	LMK-1	Lane # 13.	LAV 7649
Lane # 7.	LAV 7649	Lane # 14.	LAV 5330

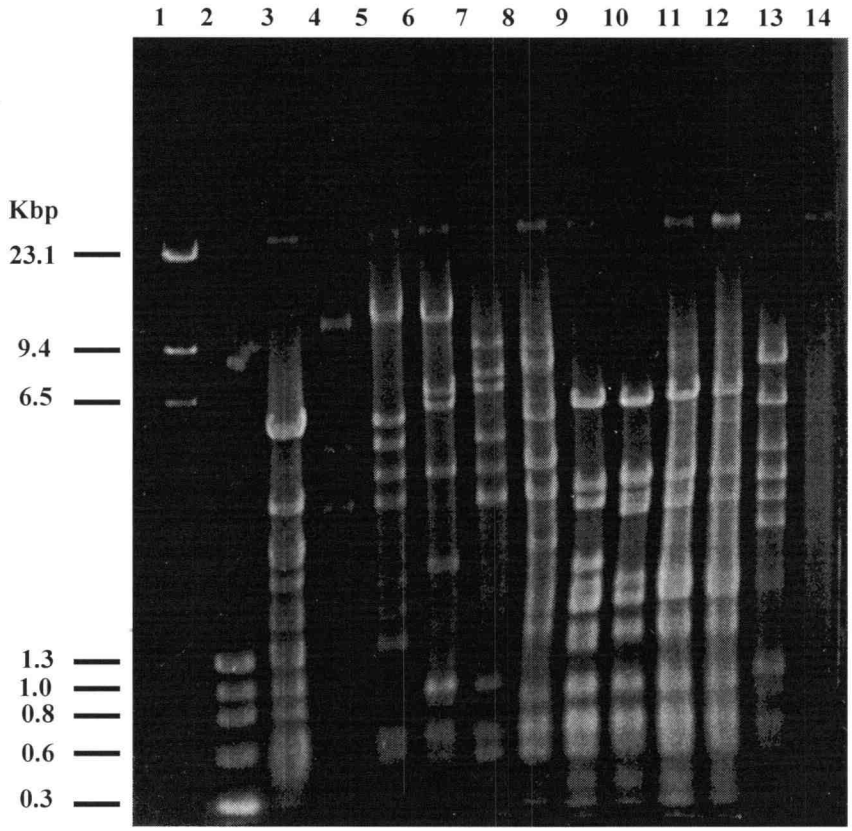

Figure 3.2. *Pst* I digest of ovine adenovirus (OAV) strains DNA.

Lane # 1.	MW Marker	Lane # 8.	RTS-42
Lane # 2.	MW Marker	Lane # 9.	OAV-6
Lane # 3.	OAV-1	Lane # 10.	RTS-151
Lane # 4.	OAV-2	Lane # 11.	475N
Lane # 5.	OAV-3	Lane # 12.	32CN
Lane # 6.	OAV-4	Lane # 13.	47F
Lane # 7.	OAV-5	Lane # 14.	OFC

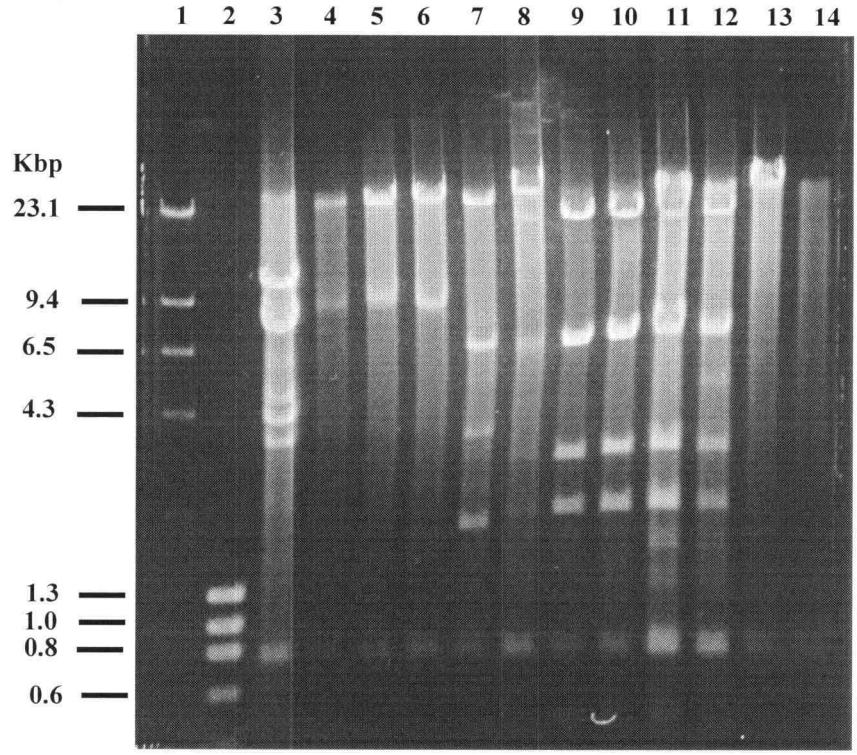

Figure 3.3. *Bam* HI digest of DNA of ovine adenovirus (OAV) strains.

Lane # 1.	MW Marker	Lane # 8.	RTS-42
Lane # 2.	MW Marker	Lane # 9.	OAV-6
Lane # 3.	OAV-1	Lane # 10.	RTS-151
Lane # 4.	OAV-2	Lane # 11.	475N
Lane # 5.	OAV-3	Lane # 12.	32CN
Lane # 6.	OAV-4	Lane # 13.	47F
Lane # 7.	OAV-5	Lane # 14.	OFC

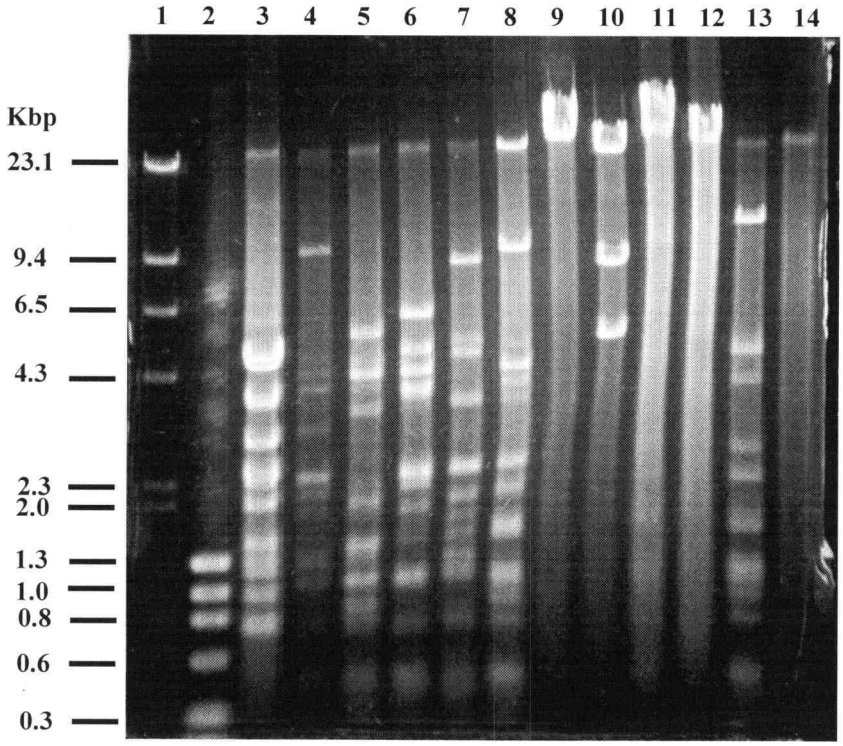

Figure 3.4. *Hind* III digest of DNA of ovine adenovirus (OAV) strains.

Lane # 1.	MW Marker	Lane # 8.	RTS-42
Lane # 2.	MW Marker	Lane # 9.	OAV-6
Lane # 3.	OAV-1	Lane # 10.	RTS-151
Lane # 4.	OAV-2	Lane # 11.	475N
Lane # 5.	OAV-3	Lane # 12.	32CN
Lane # 6.	OAV-4	Lane # 13.	47F
Lane # 7.	OAV-5	Lane # 14.	OFC

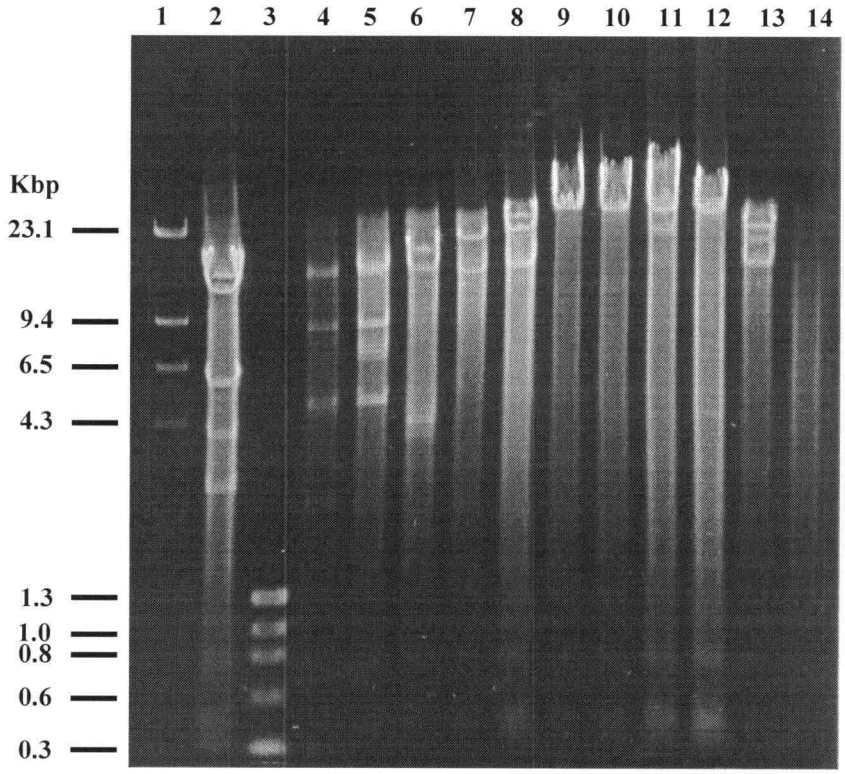

Figure 3.5. *Eco* RI digest of DNA of ovine adenovirus(OAV) strains.

Lane # 1.	MW Marker	Lane # 8.	RTS-42
Lane # 2.	OAV-1	Lane # 9.	OAV-6
Lane # 3.	MW Marker	Lane # 10.	RTS-151
Lane # 4.	OAV-2	Lane # 11.	475N
Lane # 5.	OAV-3	Lane # 12.	32CN
Lane # 6.	OAV-4	Lane # 13.	47F
Lane # 7.	OAV-5	Lane # 14.	OFC

DISCUSSION

Digestion of ovine and llama adenoviruses species with the four restriction enzymes resulted in different RE band patterns which was attributed to their genetic differences organization. These differences were expressed as variations in number and sizes of RE bands produced by each of the enzymes. Also, some strains showed differences in specificity to an individual enzyme i.e., they did not produce any bands and remain uncleaved. These differences can be used to characterize species differences within the group.

The most useful RE, cleaving all the ovine adenovirus strains and producing different RE patterns, was found to be *Pst* I. Digestion of all the recognized and untyped ovine adenovirus strains with this RE (*Pst* I) resulted in distinct patterns, except for isolates 475N and 32CN. These two serologically identical isolates showed an identical RE pattern with this enzyme. Also isolate 47F, which is serologically identical[29] to RTS-42, produced close, but not identical, RE patterns. The US isolate RTS-151, which is related serologically to OAV-6 by one-way cross but not by reciprocal neutralization, produced different numbers and sizes of bands when compared to OAV-6. This supports the concept that serologically similar[29] strains could generally produce similar RE maps and partially-related serotypes produce dissimilar RE patterns. Digestion of ovine adenovirus RTS-151 (related to OAV-6) and RTS-42 (OAV-5) and other selected ovine adenovirus isolates with this RE, also produced more distinctive bands in a limited study of their RE patterns.[23]

The second most successful restriction enzyme was *Bam* HI which digested all the strains except isolate 47F. This RE, although it did not produced many fragments for all viruses, but yielded bands were very visibal and distinct. Isolate 475N and 32CN (serologically identical but distantly related to OAV-6 and RTS-151) produced RE patterns which were similar to OAV-6 and isolate RTS-151. Considering the RE patterns produced by this enzyme for isolates 475N, 32CN, RTS-151 and OAV-6, it is evident that all these viruses possess a genetically-related genome. However, it is quite clear from their serological profile that these four strains, although genetically related, are different antigenically. In addition, isolate 47F, which is serologically identical to OAV-5 and isolate RTS-42, remain uncleaved by *Bam* HI while both of OAV-5 and RTS-42 produced distinct RE patterns. This has been confirmed in another study[23] where this RE demonstrated fewer RE bands for all the ovine adenovirus species and OAV strain RTS-42 produced RE fragments. These observations clearly show the disadvantages of RE digestion as the technique does not always define differences, as expressed in antigenic studies.

Restriction enzyme *Hind* III also demonstrated differences in genomic organization of recognized OAV and selected OAV isolates. All the OAV species digested showed distinctive RE fragments between themselves, except for the isolates 475N, 32CN and OAV-6, which remain uncleaved. Again it was demonstrated that serologically identical strains (475N and 32CN) could produce similar RE pattern and also OAV-6, which is related antigenically to these viruses, followed the same pattern. However, RTS-151 which is also antigenically related to above-mentioned strains, did not follow their restriction reaction.

Restriction enzyme *Eco* RI was shown to be similar in cleavge as *Hind* III but it produced fewer bands than *Hind* III. Some of the OAV species remain uncleaved regardless of their antigenic relationship. Isolate 475N, 32CN (antigenically identical), RTS-151 and OAV-6 (antigenically related) followed the identical RE patterns and remain uncut. Isolate 47F followed the same RE pattern of antigenically related OAV-5 (produced bands) while RTS-42, which is also related to them, did not produced any band.

Llama adenoviruses LA 7649 and LA 5330, which are antigenically distinct species, showed substantial differences when digested with restriction enzymes *Pst* I, *Bam* HI, *Hind* III and *Eco* RI. Restriction enzyme *Pst* I produced more RE fragments than other enzymes. This restriction enzyme was shown to be more successful with these two llama adenoviruses than with ovine adenoviruses in the current study. It was also observed that *Bam* HI produced less RE fragments for llama adenoviruses than was shown for ovine adenoviruses. Restriction enzyme *Eco* RI did cleave both strains of llama adenoviruses but it did not cleave all the ovine adenovirus species.

Results of this study can be explained by the fact that, by serologically defining differences between species of a virus group, there is a relatively broad range of antibody titres which do not detect minor antigenic differences. However, with RE reactions, minor differences in restriction sites can demonstrate a greater degree of variations in a virus isolate. Moreover, there is no agreed upon criterion or standard which establishes a guideline to gauge a particular magnitude of difference in RE patterns by which a virus species can be defined. Will it be possible to establish a standard RE pattern for a specific virus using a certain number of restriction enzymes?. More research in this field

including testing other restriction enzymes which can elaborate more differences may give an answer to this problem. Only further studies will show the usefulness of endonuclease reactions in demonstrating distinctiveness within a virus group. One could speculate that the ovine adenovirus group, and possibly adenovirus of all species, are composed of multiple differences in their genome. These differences may be defined by their RE patterns or by their antigenic profile, and these two methods of demonstrating distinctiveness may vary independently from each other.

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CHAPTER IV

Serological Characterization of Ovine Adenovirus Isolates 475N, 47F, Bovine Adenovirus Isolate 32CN and Llama Adenovirus Isolate 7649.

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ABSTRACT

Ten adenoviruses from sheep, one bovine and one llama were studied by virus neutralization test to determine their degree of antigenic relationships to recognized prototype viruses. Prototype viruses included recognized species of ovine adenoviruses as well as ovine adenovirus isolates RTS-42 (a member of OAV-5 species) and RTS-151 (a member of OAV-6 species). Unclassified adenoviruses that were compared to the prototypes were isolated from different locations within Oregon and were represented by bovine adenovirus isolate 32CN, ovine adenovirus isolate 475N and 47F and llama adenovirus isolate 7649. Reciprocal virus neutralization tests were performed and the degree of antigenic similarities, i.e., species differentiation, was determined by criteria established by the International Committee for the Nomenclature of Viruses. Isolates 32CN and 475N were antigenically identical and were not neutralized by any of the typing serum. They are candidates for a new species of ruminant adenoviruses. Isolate 47F was shown to be a member of OAV-5 species while the one llama virus strain represents a newly recognized species for this animal.

INTRODUCTION

The study of adenoviruses has drawn much attention since the first human adenovirus was isolated in 1953.[59] In humans, these agents produce a wide variety of disease syndromes associated with infection of the upper and lower respiratory tract,[12, 31] gastrointestinal tract,[9, 69] and ocular tissue.[46] Adenoviral infections in many animal species, as well as in humans, have generally been associated with a variety of disease symptoms

including: pharyngitis, bronchitis, bronchiolitis, conjunctivitis, keratoconjunctivitis, pneumonia, and pneumoenteritis.

Naturally-occurring bovine adenovirus (BAV) infection usually causes a mild upper respiratory disease or conjunctivitis. Bovine adenovirus species 3 can cause a mild pneumoenteritis in calves.[43] Bovine adenovirus species 2 has been shown to cause *in-utero* infection in sheep.[7] Bovine adenoviruses also have been isolated from calves with hemorrhagic gastroenteritis.[65] Canine adenoviruses cause hepatitis and laryngotracheitis.[4] Equine adenovirus (EAV) has been associated with pneumonia in foals[44] and with fever in race horses.[64] Ovine adenoviruses (OAV) and caprine adenoviruses cause a wide variety of signs associated with respiratory and alimentary tract infection.[25] Finally, llama, as a newly studied animal species, appears to have its own adenoviruses (LAV) that causes respiratory and alimentary tracts disorders. To date, only one llama adenovirus has been discovered and the virus is untyped.

The purpose of the current study was to characterize the antigenic profile of four adenovirus isolates by the virus neutralization test. All four viruses were recovered in Oregon: one adenovirus was isolated from the nasal cavity of a calf (32CN), two ovine adenoviruses (475N, 47F) were isolated from lambs with respiratory and enteric disease signs[67] and the llama adenovirus was isolated from feces of a young llama (cria) with diarrhea.

LITERATURE REVIEW

Adenoviral Classification:

Rowe and coworkers first isolated a cytopathogenic agent from human adenoids and proposed the term Adenoid Degeneration Agent in 1953.[59] Hilleman and Werner isolated these viruses from patients with acute respiratory illness and used the term Respiratory Illness Agent.[31] Huebner and colleagues in 1954, proposed the term Adeno Pharyngeal Conjunctival agents as the group name.[34] In 1956 Enders proposed adenoviruses as the group name for these viruses.[17] Lastly, the International Committee on the Taxonomy of Viruses, a virology section of International Association of Microbiological Societies, established the formerly genus adenovirus into the family *Adenoviridae*. This family is represented by two genera which consists of Mastadenovirus (adenovirus of mammals) and Aviadenovirus (adenovirus of avians).[20]

Characteristics of Adenoviruses:

The characteristics of adenovirus have been defined by Ginsberg,[27] Huebner *et al.*,[34] and Willner.[74] Adenoviruses from all species are nonenveloped, icosahedral, 700-800 Å in diameter, of a density of 1.34 g/ml in CsCl, and of a particle weight of about 175 x 10^6 daltons. All adenovirus contain linear double-stranded deoxyribonucleic acid (DNA) of 29 x 10^6 daltons molecular weight (30-37 Kilobase pair).[21,24] The icosahedral protein shell (capsid) has a 5:3:2 cubic symmetry pattern and consists of 240 hexons (each bound to six neighbors) and, at the apices, 12 pentons (each bound to 5 neighbors) which carry fibers; a fiber on each penton.[28] The hexons are polygonal prisms with a

central hole.[72] The pentons, which are more complex, consist of similar polygonal base with a fiber attached. The fibers vary in length (100 - 310 Å) and are 20 Å in diameter. The fibers have a terminal knob measuring 40 Å in diameter.[48]

Adenoviruses are resistant to the action of lipid solvents, such as ether,[34] chloroform,[19] fluorocarbons,[73] and deoxycholate[32] indicating that the viruses lack essential lipid. These agents are acid stable[30] (with few exception i,e., some strains are sensitive to pH 3[40], Babar,S. *et al.,* unpublished data, 1995) and, in fact, are more stable at acid than alkaline pH.[73] They are also relatively stable in homogenates of infected cells.[16]

Adenoviruses replicate in the epithelium or epithelial-like cells of cell cultures.[26] Adsorption of these viruses to susceptible host cells is relatively slow. With species 2 virus of human origin, approximately 70% of the viruses are adsorbed in six hours at 36 C. It is assumed that 12 hours are needed for maximum adsorption.[47]

Nucleic acid replication occurs in the nucleus. Protein is synthesized in cytoplasm and then migrates to the nucleus where the virus is assembled.[22] The assembly of viral subunits is very inefficient with only 10-15% of the viral DNA and protein incorporated into viral particles. The remaining nucleic acid and protein become parts of the intranuclear inclusions.[29] Both types A and B intranuclear inclusions are formed by various species of mammalian and avian adenoviruses.[42] The cytopathic effect of adenoviruses in susceptible cells *in vitro* is characterized by rounding and clumping of the cells[31, 60] and detachment from the surface.[49, 57, 68] These viruses also invoke an enhanced utilization of glucose in the cell cultures leading to an increase in organic acids. Therefore, adenovirus-infected cell cultures become more acidic than noninfected cultures.[23, 34]

Adenoviruses contain several antigens. By using a variety of serological techniques, group-specific, and subgroup-specific determinants can be observed from the hexon polypeptide. Both group-specific and subgroup-specific determinants can be detected from the penton base protein, while subgroup-specific and species-specific determinants can be found on the fiber.[48, 49] Formerly, the hexons, pentons, and the fibers were called antigens A, B, and C, respectively.[27] The hexon antigen is usually distinguished by complement-fixation[52, 60] or gel diffusion test,[3] and the presence of group-specific antigen is a required criterion for inclusion of viruses into the adenovirus group.[58] Exceptions to this rule have been shown for avian adenovirus by Ginsberg,[27] and subgroup 2 bovine adenovirus by Archetti and Horsfall.[5] The penton particles have been confirmed to be identical with cell-detaching factor,[57] early cytopathic factor,[91] and toxin-like material.[18, 37] Human adenoviruses species 1, 3, 7, 8, 12, 14, 16, 18, 21, 24, 31,[66] simian adenoviruses species SV7, SV20, SV33, SV34, SV37, SV38,[35] avian adenovirus (CELO),[62] infectious canine hepatitis virus,[61] and bovine adenovirus species 3 strain WBR1 [13] are oncogenic in newborn experimentally-infected hamsters. Human adenovirus species 12 is also oncogenic for suckling mice, mastomys,[55] and rats.[33]

Human Adenoviruses:

There are now 47 distinct antigenic species of human adenoviruses.[63] Infection has been associated with a wide variety of disease syndromes, including conjunctivitis, pharyngitis, keratitis, bronchitis, bronchiolitis with pneumonia, cystitis and enteritis.[43, 50, 54] Infection is usually mild but is much more severe in children and military recruits.[10] Likewise, different adenovirus species vary in their virulence with some strains more consistently inducing severe disease.[14, 51]

Ovine Adenoviruses:

Currently, six antigenic species of ovine adenoviruses and one species of caprine adenoviruses have been described.[25] 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 varies from 60 to near 100 percent. Ovine adenoviruses are frequently isolated from apparently clinically normal lambs as well as from lambs with a history of enteric and respiratory disease. Likewise, these viruses have been shown 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. Like BAV, some species of OAV have been shown to produce a viremia in the dam resulting in fetal disease.[40]

It is now apparent that interspecies transmission of BAV, OAV and CAV can occur. Sheep have been shown on several occasions to be naturally infected with BAV.[50] It also can be said 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.[38]

Darbyshire and Pereira (1964) suggested that sheep were naturally infected with adenoviruses.[15] But apart from a preliminary report by McFerran *et al.* in 1969,[45] there was no previous report of isolation of sheep adenoviruses.

Adenovirus group-specific antigens were detected in sheep and goats by Darbyshire in the United Kingdom. Serum from different animals including sheep and goats was tested in parallel with a human adenovirus species 5 antigen using the agar gel immunodiffusion (AGID) test. Results showed that 1/103 sheep and 33/50 goats had the precipitating antibodies against adenovirus group antigen.[15]

In 1983, two serologically distinct species of adenoviruses were isolated from lambs assembled at a ram testing station in the Central United States.[39] One isolate, RTS-151, was pathogenic for colostrum-deprived lambs and produced clinical signs of respiratory tract disease and severe pathologic changes.[40] In cross-neutralization tests, one of these viruses, RTS 42, was typed as OAV species 5[1, 39], while RTS 151 was unrelated to OAV species 1 through 5 or to the BAV species 1-8. However, RTS 151 did possess the adenovirus group-specific antigen as demonstrated by agar gel precipitation test. Further agar gel immunodiffusion tests showed that a common antigen was shared between BAV 3, RTS 42 and RTS 151 isolate.[40] This virus was later typed as OAV species 6 strain RTS 151.[1]

Llama Adenovirus:

Little is known about llama adenoviruses, the llama is a relatively newly-studied animal species. The work towards the investigation of viruses in llama has been started at Oregon State University. Mattson and coworkers[6] have recently identified an adenovirus in llama with diarrhea and designated the isolate as LAV 7649. This adenovirus, however, has not been yet typed. According to Mattson, there are several other isolates that are now being characterized; one of those was isolated in Michigan.

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[6] Mattson *et al.*, 1995: Unpublished Data.

MATERIALS AND METHODS

Media:

The medium used for growth and maintenance of cell cultures and for dilution of virus and serum was Minimal Essential Medium (MEM) with Earle's salts.[7] In addition, MEM used in cell cultures contained 100 units per ml penicillin and 100 μg per ml streptomycin sulfate. Growth medium for cell cultures contained 10 percent fetal bovine serum, while maintenance medium contained 5 percent fetal bovine serum. Serum used for cell cultures was shown to be free of antibodies to the viruses being tested. A Tris-EDTA (TE) solution was used to resuspend purified virus and was composed of Tris (0.01M), EDTA (0.001M), and NaCl (0.1 M) at pH 7.5.

Cell Cultures:

Ovine fetal corneal cells (OFC) and llama kidney cells (LMK-1) were used in all virus neutralization tests. Llama kidney cell cultures were propagated from a 4-month-old llama. Primary cell cultures were prepared by the trypsin digestion method. Ovine fetal corneal cells (OFC) were received from the National Animal Disease Service Laboratory.[8] Stock cultures of primary cells and cell lines were transferred with a

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[7] Gibco BRL, Life Technologies Inc., Grand Island, N.Y. Catalogue No. 410-1600 EL.

[8] National Veterinary Service Laboratories. PO Box 844, Ames, Iowa 50010.

Hepes-EDTA-trypsin solution pH 7.5 (0.35 gram percent Hepes, 0.072 gram percent glucose, 0.022 gram percent KCl, 0.759 gram percent NaCl, 0.14 gram percent sodium phosphate, 0.02 gram percent EDTA, and 100 mg percent trypsin). Transferred cells were used to produce either additional stock cultures or cultures in microtiter plates.

Virus Source:

Ovine adenovirus, prototype species 1-6, strain RTS-42 and strain RTS-151, were received from Dr. Howard Lehmkuhl.[9] Ovine adenovirus strains OAV 47F, and OAV 475N were isolated in Oregon from lambs.[67] Bovine adenovirus strain 32CN (BAV 32CN) was isolated from a 4-month-old calf with conjunctivitis.[10] Llama adenovirus strain 7649 (LAV 7649) was isolated from the feces of a 2-week-old cria with diarrhea.[11] Stock virus was allowed to replicate in OFC (ovine adenoviruses) and LMK-1 cells (llama adenovirus).

Virus (10 ml suspension) was inoculated upon cell culture monolayers in 490 cm^2 roller bottles. Virus was adsorbed for 4 hours at 37 C after which the maintenance medium was added. Cultures were incubated at 37 C for 24 hours after cytopathic effect (CPE) involved nearly 100 percent of cells (usually 5 to 7 days). Cells were scraped

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[10] Donald Mattson, DVM, PhD., Personal Communication. College of Veterinary Medicine Oregon State University, Corvallis, OR 97331.

[11] Idem footnote # 10.

from the flask, subjected to ultrasonic disruption for 45 seconds and clarified by centrifugation at 3000 times gravity for 20 minutes. The supernatant fluid was placed in vials and frozen at minus 30 C until used. Virus was titered in 10-fold dilution steps in 96- well flat bottom microtiter plates.[12] Titer of the virus was calculated by the Reed-Muench formula.[41]

Antiserum Production (Anti 475N, 32CN, 47F, RTS-42, RTS-151 and LAV 7649):

Virus replication for production of antiserum in rabbits was similar to that which has been described previously. When culture flasks were ready to harvest, the cells were scraped from the flask. The infected cells were centrifuged 2000 times gravity for 20 minutes. The cell pellet was washed once in TE buffer and recentrifugated. The pellet was resuspended in one tenth of the original volume in TE buffer. The cells were subjected to ultrasonic disruption for 1 minute at 4 C. An equal volume of trichloro-trifluoroethane (TCTFE) was added to the preparation and the product was mixed in a blender for 2 minutes. The mixture was centrifuged 2000 times gravity for 15 minutes, and the supernatant fluid removed. Ten milliliters of TE solution was added to the interphase and the product was blended and centrifuged again, the two supernatant fluid layers were combined. Twenty-five milliliter of virus suspension was layered on a discontinuous cesium chloride gradient bilayers (top layer, 6 ml with a density of 1.2 g per ml and bottom layer, 8 ml with a density of 1.4 g per ml). The preparation was

[12] Catalogue No. 25861, Corning Glass Works. Corning, New York 14831.

centrifuged 83,000 times gravity for 90 minutes at 4 C in a SW 28 rotor. The opaque bands at the 1.4 g per ml interface from all tubes were removed and pooled. The bands were mixed in 1.33 g per ml cesium chloride in TE solution to a total volume of 38 ml. The preparation was centrifuged 83,000 times gravity for 36 hours at 4 C. The opaque band was removed by side puncture, confirmed to be 1.3-1.34 g per ml density, and dialyzed against physiological buffered saline (pH 7.5) for 24 hours. The final purification product was examined by negative staining in an electron microscope to assure the presence of virus. The preparation was divided into 4 aliquots. Aliquot one was mixed with an equal amount of Freund's Complete Adjuvant and injected subcutaneously (four sites) and intramuscularly (one site) in a New Zealand white rabbit. Each different adenovirus prototype or untyped virus was injected into different rabbits. 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 subcutaneously (4 sites) on days 7, 14, and 21. The rabbit was exsanguinated by cardiac puncture on day 35. Serum was separated and heat-inactivated at 56 C for 30 minutes after which aliquots (3 ml each) were stored at minus 30 C. Antisera to ovine adenovirus prototype species 1-6 were received from another source[13].

Virus Neutralization Test:

Antigenic distinctiveness among the different adenoviruses was determined by the virus neutralization (VN) test. Antiserums to the viruses were thawed and diluted in 2-

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[13] Idem footnote # 1.

fold steps in 50 µl MEM in flat bottom microtiter plates.[56] Each dilution series was performed in triplicate beginning with a 1:8 dilution of serum. One hundred medium tissue culture infectious doses of virus in 50 µl of MEM was added to each well containing the diluted serum. Initial serum dilution was now 1:16. Plates were incubated at 37 C for 60 minutes after which 50 µl of MEM containing 5×10^5 cells was added to each well. Finally, one drop of sterile mineral oil was added to each well to prevent drying. The plates were placed in a CO_2 incubator (2.5% CO_2 concentration) and incubated at 37 C. Cultures were examined for CPE after 7 days. Serum end-points were determined by the Reed-Muench formula.[41] One antibody unit (AbU) was defined as that dilution of antiserum which neutralized 100 $TCID_{50}$ of virus. Accordingly, the end-point titer of a homologous antiserum contained one AbU, two AbU would be twice as concentrated etc.

RESULTS

Virus neutralization tests indicated some cross-neutralizations occurred in both intra-species and inter-species of animals. A complete summary of cross-neutralizations is presented (Table 4.1).

Ovine Adenovirus Prototypes 1 to 6:

All the ovine adenovirus prototype species demonstrated complete uniqueness among themselves and with llama adenovirus virus isolate. Although some minor

antigenic similarities were shared, their reciprocal antibody titer differences were > 16 antibody units (AbU) by reciprocal virus neutralization (VN) tests.

Bovine Adenovirus Isolate 32CN:

Antiserum to 32CN neutralized isolate 475N with one AbU. The 32CN antiserum also neutralized OAV-6 and RTS-151 with > 19 and > 13 AbU, respectively (indicating a moderate antigenic relationship between these virus isolates). In reciprocal VN studies, antiserum to 475N neutralized 32CN virus equally i.e., ≤ 1 AbU while antiserum to OAV-6 and RTS-151 neutralized 32CN with > 16 and 45 AbU, respectively. There was a low-level neutralization between 32CN antiserum and LA7649 but > 16 AbU were required for neutralization.

Ovine Adenovirus Isolate 475N:

Antiserum to 475N neutralized the 32CN virus with ≤ 1 AbU. Using this antiserum, it took 65 AbU to neutralize OAV-6 and 8 AbU to neutralize RTS-151. In reciprocal VN studies, OAV-6 did not neutralize 475N i.e., > 16 AbU and RTS-151 antiserum neutralized 475N with 20 AbU.

Ovine Adenovirus Isolate 47F:

Antiserum to OAV 47F neutralized a number of viruses i.e., OAV-5 with 1 AbU, RTS-42 with 2 AbU, OAV-3 with 8 AbU, OAV-4 with 75 AbU, and OAV-2 with 127 AbU. In reciprocal studies, the 47F virus was neutralized by antiserum to OAV-5 and

RTS-42 with < 1 AbU. This isolate was not neutralized (reciprocally) by antiserum to OAV-4, OAV-3 and OAV-2 i.e., > 16 AbU were required if neutralization occurred.

Llama Adenovirus Isolate 7649:

Antiserum to LA 7649 neutralized the 475N isolate with 255 AbU and 32CN with 75 AbU. In reciprocal SN tests, antiserum to 475N did not neutralized LA7649 and antiserum to 32CN neutralized LA7649 with 211 AbU.

Table 4.1 Reciprocal virus neutralization of ovine and llama adenoviruses.

VIRUS	Antiserum Against											
	OAV1	OAV2	OAV3	OAV4	OAV5	OAV6	475N	32CN	RTS42	RTS151	47F	LA7649
OAV1	NT											
OAV2	NT	1420	88	44					180		44	
OAV3	NT	53	1420						600		710	
OAV4	NT	27		2820					210			
OAV5	NT				850				9550		2820	
OAV6	NT					340	44	600		1700		
475N	NT						2840	≥11200		340		44
32CN	NT						5630	≥11200		150		150
RTS42	NT	27	27	75	≥1120				≥11200		3390	
RTS151	NT						340	850		6770		
47F	NT			38	1700				≥11200		5630	
LA7649	NT							53	340			≥11200

The numbers indicate reciprocal of the serum dilution. Titer that is equal or higher than 1:22 is regarded positive. Highlighted cells homologous titers. Blank cells are negative i.e., titer ≤1:22. NT = not tested.

DISCUSSION

Antigenic distinctiveness of adenovirus species is determined by the virus neutralization test. A test virus is determined to be of the same species as a prototype virus if less than or equal to 16 antibody units of reference antiserum to the prototype virus neutralizes a test virus. Using standard virus neutralization procedures, one antibody unit is defined as the most dilute concentration of antiserum that neutralizes 100 $TCID_{50}$ of homologous virus. Virus neutralization also must be demonstrated in a reciprocal fashion with 16 antibody units or less of heterologous antiserum being capable of neutralizing the prototype virus.[71]

Using the above parameters, this investigation has shown that ovine adenovirus OAV-5 and RTS-42 are of the same antigenic species as Oregon isolate OAV 47F. It has been shown previously by other investigators that prototype OAV-5 and isolate RTS-42 are identical.[1]

This research also has shown that Oregon isolate OAV 475N is of the same antigenic species as Oregon isolate 32CN i.e., there was a reciprocal neutralization of $\leq$ 16 AbU. Although both of these viruses were isolated from two different animal species, this study has again shown that adenoviruses can infect across species of animals[6, 7]. All the prototypes of ovine adenoviruses were available for the current comparison. Because of a problem with the production of antiserum to prototype OAV-1, it was not possible to test this virus in a reciprocal fashion. However, the OAV-1 prototype species was not

neutralized in a one-way VN test by antiserum to other OAV species and the test viruses under consideration.

In addition to confirming that 32CN and 475N were of the same antigenic species,[75] it is apparent that these viruses are closely related to RTS-151 and OAV-6. There appears to be a cluster of antigenic similarity between 32CN, 475N, RTS-151 and OAV-6. Other studies have alleged that OAV-6 and RTS-151 are of the same antigenic species. However, their own data indicates they are not within the 16 AbU as defined by the report from the adenoviruses study group.[1] The present study confirms that OAV-6 and RTS-151 are antigenically related but are of a different species.

Early in the period when adenoviruses were first characterized, it was noted that these viruses were relatively species-specific both to the natural host and to cell cultures derived from the natural host.[36, 58] While this is a vague, indefinable categorization and not suitable for precise taxonomic purposes, it is an important biological characteristic which appears to hold true for all group members even some 30 years after these viruses were characterized. These viruses have been shown repeatedly to replicate and produce disease only in the natural host or closely related species. Likewise, adenoviruses generally have the ability to undergo continuous propagation in cell cultures derived only from the natural host or its close relatives. With large inoculum doses, the virus may induce a cytopathic effect in cell culture derived from an alien host, but such infection is generally abortive.[58]

The current research has reconfirmed the host specificity nature of adenoviruses by showing that adenoviruses from diverse species do not share common major antigens.

While this observation has been noted with most species previously, this research confirms the results of an initial study[75] where the antigenic nature and cell culture specificity of a llama adenovirus have been studied. This study also confirms the results of a previous study[75] that determined the serological profile of llama adenovirus and defined it's antigenic relationship with ovine adenoviruses. While additional adenovirus from the llama may be recovered in the future, it has been shown that LAV isolate 7649 was distinct antigenically from other viruses characterized in this investigation.

Adenoviruses from such diverse species as equine, llama and members of the *Bovidae* family (bovine and ovine) were shown not to share major neutralizing antigenic components.[75] However, due to the complex antigenic nature of the adenoviral capsid (where several antigens may be involved in the virus neutralization reaction), some minor antigens were detected between adenoviruses from different species of animals.

In the current study, two species of animals (bovine and ovine) are closely related anatomically and physiologically and both are classified in the *Bovidae* family of animals.[11] This research has shown that bovines and ovines appear to be able to be infected with isolate 32CN (a bovine isolate) and 475N (an ovine isolate). Further, previous studies conducted by others have shown that sheep can be infected with BAV species 2 and species 7.[2, 6]

The antigenic composition of human adenoviruses has been studied more extensively than any other species. Several human adenoviruses share common antigenic determinants even though they are classified as different species. It is not uncommon to have several members of the 47 species to be related antigenically in a variable fashion.

Some species are so closely related that infection is difficult to diagnose by serologic testing, i.e., when a person becomes infected with one virus, an antibody response ensues against the homologous and heterologous species (antigenically related species) of virus (heterotypic antibody response).[70] This observation has an epidemiological significance in human disease. In addition, knowledge of this immunologic response pattern also may have implications in possible candidate species for vaccine production. The number of strains of virus for incorporation in a vaccine is obviously limited. Accordingly, in selecting such a virus, it would be appropriate to use a species of virus with a broad heterotypic antigenic profile for the species of animal in question.

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CHAPTER V

CONCLUSIONS

Respiratory tract diseases (RTD) among young lambs are very common and result in major economic losses to this industry. Numerous microbial agents have been associated with this disease syndrome and, among them the role of viruses is well established. However, the etiologic significance of adenoviruses in initiating RTD in sheep has not been fully understood. Several types of investigative studies including virus isolation, experimental infection of young lambs, serological characterization, seroepidemiological surveys and other related studies have been conducted in many countries in an attempt to clarify this question.

Adenoviruses have been isolated from sheep with a variety of disease syndromes but they are believed primarily to be involved in respiratory tract or gastrointestinal tract disease or a combination of both. These agents have been isolated from healthy sheep as well as from those showing signs of pneumonia, enteritis or pneumoenteritis. Although adenoviruses are said to be host-specific, it has been shown on a few occasions that infection can take place across species of animals.

In the present study, an unclassified isolate of ovine adenovirus was further characterized. This isolate, 475N, which was isolated from a sheep in Oregon with respiratory tract disease signs, was shown to be a new species of virus for this animal (in addition to the six OAV species currently recognized). This new strain has a potential of producing pneumonia in young lambs. Clinical signs, gross pathology, and

histopathology produced by this strain are suggestive of its role in causing RTD. However, it is noted that RTD is a combination of a variety of diverse factors including initial virus infection.

The restriction endonuclease reaction patterns of isolate 475N are different from other recognized ovine adenovirus species. Although these restriction endonuclease maps are not conclusive, they are suggestive of its different genetic composition from other recognized ovine adenoviruses. However, this strain showed some similarity to OAV-6 and the US variant of OAV-6, RTS-151. Antigenically, 475N showed an identical reciprocal antibody titre to a bovine adenovirus isolate 32CN. However, it was shown to differ antigenically from other recognized ovine adenovirus types. It is apparent that isolate 475N shared some antigens with prototype species, OAV-6 but the antibody ratio for its dissimilarity meet the standards established by the International Committee on Taxonomy and Classification of Viruses. In this study, characterization of adenovirus isolate 475N from Oregon sheep, shows that this strain represents a new species of adenovirus in sheep.

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