Fifteen adenoviruses from cattle, sheep, horses, and llamas were studied by virus neutralization to determine their degree of antigenic similarity. Prototype viruses included bovine adenoviruses species 1-8, ovine adenoviruses species 5 and 6, and equine adenovirus species 1. Unclassified viruses that were compared to the prototype viruses were isolated from different locations within Oregon and were represented by bovine isolate 32CN, ovine isolates 47F and 475N, and llama isolate 7649. Reciprocal virus neutralization tests were performed and the degree of antigenic similarity, i.e., species differentiation was determined by criteria established by the International Committee for the Nomenclature of Viruses.

The study showed that many of the adenoviruses, both prototype and unclassified, shared minor antigenic components with each other. Prototype viruses possessed major antigenic differences and, as previously demonstrated by other investigators, should be classified as separate virus species. Bovine adenovirus isolate 32CN was shown to be of the same species as ovine adenovirus isolate 475N, but neither isolate was similar to any of the prototype virus species studied. Ovine adenovirus isolate 47F was shown to be of the same species as ovine adenovirus species 5 strain RTS 42. Llama adenovirus isolate 7649, while sharing minor antigens with different viruses from cattle and sheep, was shown to be a distinct species. This represents the first species of adenovirus from llama.
ANTIGENIC COMPARISON OF BOVINE, OVINE, EQUINE, AND LLAMA ADENOVIRUSES

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ANTIGENIC COMPARISON OF BOVINE, OVINE, EQUINE, AND LLAMA ADENOVIRUSES

I. INTRODUCTION

The study of adenoviruses has drawn much attention since the first human adenovirus was isolated in 1953. In human, these agents produce a wide variety of disease syndromes associated with infection of the upper and lower respiratory tract, gastro-intestinal tract, and ocular tissue. 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, and 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. Bovine adenovirus species 2 has been shown to cause in utero infection in sheep. Bovine adenoviruses have also been isolated from calves with hemorrhagic gastroenteritis. Canine adenoviruses cause hepatitis and laryngotracheitis. Equine adenovirus (EAV) has been associated with pneumonia in foals and with fever in racehorses. Ovine adenoviruses (OAV) and caprine adenoviruses cause a wide variety of signs associated with the respiratory and alimentary tract infection. Finally, llama, as a newly studied animal species appears to have its own adenovirus (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 adenoviruses by the virus neutralization test. All four viruses were recovered
in Oregon: one adenovirus was isolated from the nasal cavity of a calf, two ovine adenoviruses were isolated from lambs and one adenovirus was isolated from a young llama (cria). Another purpose of this investigation was to confirm the antigenic profiles of available adenoviruses. The outcome of this study is expected to facilitate the diagnosis of animal adenoviruses, as well as to provide direction for selection of a vaccine candidate strain should it be considered necessary and feasible. Due to some limitations, this study did not involve swine adenoviruses, caprine adenoviruses and ovine adenoviruses species 1-4.
II. LITERATURE REVIEW

Adenoviral Classification

Rowe and coworkers first isolated a cytopathogenic agent from human adenoids and proposed the term Adenoid Degeneration agent in 1953. Hilleman and Werner isolated these viruses from patients with acute respiratory illness and used the term Respiratory Illness agent. Huebner and colleagues in 1954 proposed the term Adeno Pharyngeal Conjunctival agents as the group name. In 1956 Enders proposed adenoviruses as the group name for these viruses. 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).

The characteristics of adenovirus have been defined by Ginsberg, Huebner et al., and Willner. 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. 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. The hexons are polygonal prisms with a central hole. 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.
Adenoviruses are resistant to the action of lipid solvents, such as ether, chloroform, fluorocarbons, and deoxycholate indicating that the viruses lack essential lipid. Adenoviruses are acid stable and, in fact, are more stable at acid than alkaline pH. They are also relatively stable in homogenates of infected cells.

Adenoviruses replicate in the epithelium or epithelial-like cells of cell cultures. 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.

Nucleic acid replication occurs in the nucleus. Protein is synthesized in cytoplasm, it then migrates to the nucleus where the virus is assembled. 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. Both types A and B intranuclear inclusions are formed by various species of mammalian and avian adenoviruses. The cytopathic effect of adenoviruses in susceptible cells in vitro is characterized by rounding and clumping of the cells and detachment from the surface. 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 acid than noninfected cell.

Adenoviruses contain several antigens. By using a variety of serological techniques, group, species, and subgroup-specific determinants can be observed from the hexon polypeptide. Both group and subgroup-specific determinants can be detected from the penton base protein, while subgroup and species specific determinants can be found from the fiber. Formerly, the hexons, pentons, and the fibers were called antigens A, B, and C, respectively. The hexon antigen is
usually distinguished by complement fixation\textsuperscript{90, 101} or gel diffusion test,\textsuperscript{6} and the presence of group-specific antigen is a required criterion for inclusion of viruses into the adenovirus group.\textsuperscript{99} Exceptions to this rule have been shown for avian adenovirus by Ginsberg,\textsuperscript{45} and subgroup 2 bovine adenovirus by Archetti and Harsfall Jr.\textsuperscript{8} The pentons particles have been confirmed to be identical with cell-detaching factor,\textsuperscript{98} early cytopathic factor,\textsuperscript{91} and toxin-like material.\textsuperscript{36, 63} Human adenoviruses species 1, 3, 7, 8, 12, 14, 16, 18, 21, 24, 31,\textsuperscript{113} simian adenoviruses species SV7, SV20, SV33, SV34, SV37, SV38,\textsuperscript{56} avian adenovirus (CELO),\textsuperscript{103} infectious canine hepatitis virus,\textsuperscript{102} and bovine adenovirus species 3 strain WBR 1\textsuperscript{23} are oncogenic in newborn experimentally-infected hamsters. Human adenovirus species 12 is also oncogenic for suckling mice, mastomys,\textsuperscript{94} and rats.\textsuperscript{54}

There are now 41 distinct antigenic species of human adenoviruses.\textsuperscript{105} Infection has been associated with a wide variety of disease syndromes, including conjunctivitis, pharyngitis, keratitis, bronchitis, bronchiolitis with pneumonia, cystitis and enteritis.\textsuperscript{74, 86, 92} Infection is usually mild but is much more severe in children and military recruits.\textsuperscript{18} Likewise, different adenovirus species vary in their virulence with some strains more consistently inducing severe disease.\textsuperscript{23, 88} Adenoviruses are ubiquitous\textsuperscript{49} and the number of species isolated and characterized is increasing with the passage of time. Animal for which adenoviruses are described includes cattle, horses, sheep, goat, pigs, birds, and mice. In recent years researchers have also isolated adenovirus from buffaloes,\textsuperscript{9} reptiles,\textsuperscript{60} and llamas.\textsuperscript{76} For the time being there are 41 human adenovirus species, 9 bovine species, 6 ovine species, 2 caprine species, 4 porcine, 1 equine, 1 murine, 14 avian species, and many other isolated but they have not yet been characterized. Adenovirus infections are primarily host specific; however, cross species infections are not uncommon among closely related, e.g., sheep and cattle.\textsuperscript{14}
Bovine Adenoviruses

Bovine adenoviruses (BAV) were first isolated in the United States. Klein et al. recovered two isolates (strain 10 and 19) from the feces of apparently healthy cattle. These isolates have been designated as species 1 and species 2, respectively.61-62

Ide and his coworkers isolated BAV species 1 from a calf with pneumonia and enteritis in Canada. Calves inoculated intratracheally or intravenously with this strain showed no clinical response, although an immune response did develop.57 BAV species 1 was also isolated from a calf with pneumoenteritis in Norway.104

Darbyshire and coworkers isolated BAV species 3 (strain WBR 1) from the conjunctiva of an apparently healthy cow in England.27 When it was inoculated in newborn calves, the virus was shown to invoke a mild clinical response characterized by pyrexia, respiratory distress, nasal and conjunctival discharges. Although diarrhea was not a prominent sign of the disease, the virus was isolated from both the respiratory and alimentary tracts.23

In calves inoculated with BAV species 1 (strain 10) and species 2 (strain 19), a mild respiratory disease was produced. Gross and microscopic tissue changes were generally similar, but less severe than those observed with BAV species 3 (strain WBR 1) infection.27 This finding suggested that species 3 is the most virulent of the first three species of bovine adenoviruses.

Researchers in Hungary isolated BAV species 4 (strain THT/62) and BAV species 5 (strain BA/65) from the nasal cavity, various organs, and intestinal contents of calves with pneumoenteritis.4,12 Bürki and coworkers also revealed that BAV species 4 and 5 were associated with enzootic pneumoenteritis in Austria.19 In Japan, Tanaka and other investigators isolated a BAV (strain Nagano) from the
blood of a 25-month-old bull with pyrexia, anorexia, and soft feces. Further studies showed that strain Nagano was serologically related to BAV species 4 (strain THT/62).

There were further reports on the discovery of adenoviruses. Rondhuis reported the isolation of BAV species 6 (strain 671130) which was recovered as a latent virus from bovine testicular cell cultures. Wilcox recovered two adenoviruses (strain KC1 and KC2) from cattle with conjunctivitis and keratoconjunctivitis in Australia. Inaba and coworkers isolated strain Fukuroi from a three-year-old cow with pyrexia, anorexia, diarrhea, and respiratory disease in Japan. Strain Fukuroi was later designated as species 7. Bartha et al. reported the isolation of BAV species 8 (strain MISK/67) from calves with pneumoenteritis in Hungary. This was shown to be partially related antigenically to BAV species 4, 5, and 6. Guenov and coworkers isolated BAV species 9 (strain Sofia 4/67) from the kidney of an apparently healthy calf in Bulgaria. Mattson reported the isolation of strain 5C from Oregon calves with pneumoenteritis. This strain was serologically related to BAV species 3 (strain WBR 1). Alieva et al. recovered an untyped adenoviruses from calves with pneumonia in Russia. Lupini et al. isolated an untyped adenoviruses from calves with respiratory symptoms: coughing, dyspnea, and fever in Italy.

On the basis of certain physical, chemical, and biological properties, Bartha suggested dividing bovine adenoviruses into two subgroups. The strains in subgroup 1 (species 1, 2, 3) possess the soluble antigen common with human adenoviruses, are inactivated at 56 °C for 30 minutes, replicate well in bovine kidney cell cultures, form a single intranuclear inclusion which is irregular in shape, and are readily isolated in the first passage of cell culture. The strains designated as members of subgroup 2 (species 4, 5, 6, 7, strain BIL) have only traces of the soluble antigen common with human adenoviruses, are partially inactivated at 56 °C for 30
minutes, replicate in bovine testicular cell cultures, form multiple intranuclear inclusions which are regular in shape, and are isolated only after a series of blind passages in cell culture.\textsuperscript{10} It was later shown, however, that heat inactivation differences between the two subgroups appear to be variable and it was suggested that this criterion be deleted for differentiation.\textsuperscript{11,74} In additional to what has been proposed by Bartha, later, BAV species 9 and BAV species 8 were added into subgroup 1 and subgroup 2, respectively.\textsuperscript{75}

**Ovine Adenoviruses**

Currently, six antigenic species of ovine adenoviruses and two species of caprine adenoviruses have been described.\textsuperscript{43} These numbers will unquestionably increase as research continues.

Ovine adenovirus (OAV) have been isolated from numerous countries, the percentage of adult animals possessing antibodies to these agents, indicating previous infection, varies from 60 to near 100 percent. 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.\textsuperscript{67}

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.\textsuperscript{86} 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.65

Darbyshire and Pereira suggested that sheep were naturally infected with adenoviruses.26 But apart from a preliminary report by McFerran et al. in 1969,81 there was no previous report of isolation of sheep adenoviruses.

Successful attempts to isolate adenovirus from sheep were made as early as 1969 by McFerran and coworkers in Ireland. On the basis of morphology and other physical and serological properties, and the fact that the viruses were isolated from the feces of diseased sheep, the authors suggested their possible role in producing pneumoenteritis in sheep.81 Using lamb kidney cultures, McFerran et al. isolated three serologically distinct OAV from feces of sheep. Two of these three isolated strains showed cross reactivity to adenovirus group antigens. Their work suggested that adenoviruses can be recovered from both healthy and diseased animals. Detection of OAV from the feces suggested their involvement in enteric infections.80

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 Immuno Diffusion (AGID) test. Results showed that 1/103 sheep and 33/50 goats had the precipitating antibodies against adenovirus group antigen.26

Sharp et al. described a new adenovirus in sheep. They showed that this new strain, 7769, was quite distinct antigenically from the three serotypes of OAV previously isolated. They were able to demonstrate that strain 7769 was not neutralized by antiserums to BAV as were the three serospecies isolated earlier. They inoculated the virus in pathogen-free lambs, and it replicated and stimulated an immunological response without producing any clinical signs. This suggested
that strain 7769 was non-pathogenic or causes disease in conjunction with other agents or factors.107

Belák and Palfi reported adenovirus infection in lambs in fattening facilities and described the epizootiology of the respiratory syndrome and suggested the disease contributing factors. Looking for the viral involvement, the workers were able to isolate an adenovirus, strain Het/3, from the nasal discharge of the affected lambs.16 In effort to confirm that the isolated Het/3 adenovirus strain was the etiological agent of respiratory disease in sheep, Belak et al. later conducted an experimental study, which covered several aspects of the disease. They concluded that the strain Het/3 is related to BAV species 2.14

Thurley and colleagues described the causes of mild pneumonia of lambs in New Zealand. According to their findings, beside bacterial, serum antibody titers to two adenoviruses were found. Two serological distinct viruses (later confirmed as OAV) were isolated from the feces and nasal secretions of sheep.112

In a seroprevalence and microbiological study of pneumonia of lambs in New Zealand, beside describing bacterial isolation and lesions, Pfeffer and coworkers reported the isolation of adenoviruses which were neutralized by antiserum to a local untyped strain of OAV (WV 757/75).93

New Zealand investigators, Davies and Humphreys, described the characterization of two strains of adenovirus which were recovered from sheep, in New Zealand over a period of 10 months. Samples were collected from nasal secretions, feces and lungs of dead animal and propagated in lambs testicular (LT) cultures. Two distinctly different adenoviruses species were isolated, neither of them agglutinated mouse, rat, guinea pig, sheep, cattle, and human "O" erythrocytes but one of the isolates agglutinated chicken erythrocytes. Both strains were serologically distinct on the basis of cross neutralization test. However, their relationship to the five established serospecies of ovine adenovirus was not determined.50
The same authors experimentally infected 3 to 4 months old colostrum-deprived lambs with an untyped OAV isolate strain (757/75). The virus which was administered intranasally and intratracheally, induced moderate clinical signs of respiratory tract disease. Pathological changes were limited to the respiratory tract. The lambs experienced a viremia as the virus was detected in the blood up to 14 days after infection. The WV 757/75, together with PI-3, has also been found to infect pregnant ewes and both the agent and antibody against it were passed to the fetuses. The newborns were shown to eventually suffer from the infection later in their life when the maternal antibody abated.

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

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. Clinically, the group which received the virus followed by the bacteria developed more severe signs of respiratory tract disease while the groups that received the virus or bacteria alone had minor signs of disease. Similarly, the number of bacteria from the group inoculated with Pasteurella haemolytica alone was low as compared to the group which received both bacteria and virus. This showed that bacteria alone was eliminated efficiently from lungs by
body defense mechanisms. This study suggested that the OAV strain WV 419/75, must be considered as another potential respiratory pathogen capable of initiating a bacterial bronchopneumonia.\textsuperscript{29}

Sharp \textit{et al.} experimented with ovine adenovirus species 4 strain 7769 and found it to be a primarily enteric virus. However, by mean of aerosol transmission, the virus was shown to replicate first in the respiratory tract followed by the alimentary tract.\textsuperscript{106}

Pálya and coworkers inoculated ovine adenovirus strain GY/14 and strain Het/3 in colostrum-deprived 3 weeks-old-lambs. They found no animal died because of infection although the clinical signs of respiratory and alimentary tracts involvement were evident. They concluded that GY/14 and Het/3 strains of ovine adenovirus were similar in being able to induce the disease. However, GY/14 strain caused more severe clinical signs of disease.\textsuperscript{88}

Pálfi \textit{et al.} studied OAV strain GY/14 and noted that acute phase of infection was manifested by signs of respiratory tract disease with associated pathologic changes. In the chronic phase of disease, signs were limited to reduced growth rate, varying degrees of anorexia and intermittent pyrexia. Pathologic changes were observed in the lungs and kidneys. Virus isolation was difficult in the chronic phase of the disease as the organ involved e.g. lung explants had to be cultured with ovine fetal cells. Lambs produced a good antibody response to the virus with the titre varying from 1:32 to 1:128 on day 17 post inoculation. The authors concluded that some lambs shed virus for prolonged periods of time for factors not completely understood.\textsuperscript{87}

In 1983, two serospecies of adenoviruses were isolated from lambs assembled at a ram testing station in Central America.\textsuperscript{66} One isolate, RTS 151, was pathogenic for colostrum-deprived lambs producing clinical signs of respiratory tract disease and severe pathologic changes.\textsuperscript{67} In cross neutralization tests, one of
these viruses, RTS 42, was typed as OAV species 5;\textsuperscript{1,66} 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 and further agar gel immunodiffusion test showed that a common antigen was shared between BAV 3, RTS 42 and RTS 151 isolate.\textsuperscript{67} This virus was later typed as OAV species 6 strain RTS 151.\textsuperscript{1}

Lehmkuhl et al. conducted a two year long seroepidemiological study on lambs for the presence of common respiratory viruses of sheep. They reported that infections due to OAV were substantial. The mean prevalence for both years of blood collection shows; 95% for OAV 5, 87.2% for PI-3 virus, 84.5% for RSV, 41.7% for OAV 6, 8.7% for BVD virus, 5.4% for BHV-1 and 3.3% for ovine progressive pneumonia (OPP) virus. Ovine adenovirus species 6 had the highest infection rate on the basis of mean of two year serum analysis i.e., 42.8% (207/484) as determined by an increase of equal to or greater than 4-fold serum antibody titre as compared from 1st to 2nd sample. The infection rates for other viruses were; 31.1% for OAV 5 (RTS 151), 15.3% PI-3 virus, 5.6% for RSV, 0.6% for BVD virus and 0.4% for BHV-1.\textsuperscript{68}

In an advanced study, Lehmkuhl and coworkers infected a group of one week-colostrum-deprived lambs with the strain RTS 42 (OAV 5) and necropsied them on different days after inoculation until 21 days PI. Virus was present in nasal secretions and lung tissues in all lambs killed between days 1-6 PI. However, they were not able to recover the virus from any other organ including intestine. Virus neutralization antibodies first appeared on day 6 PI and were high in the serum of lambs killed on day 12 PI but the titers decreased in serums collected on day 21 PI. Because the virus was not isolated from any other organ except the lungs, the authors suggested that RTS 42 is a respiratory virus. The presence of virus in feces can be explained by observation that RTS 42 is acid resistant and
may have replicated in the respiratory tract and passed through the digestive tract.  

Dubey and Sharma reported the presence of OAV 1 in India. The virus was isolated from the sheep with pneumoenteritis. Nasal and fecal samples were inoculated in embryonic lamb kidney (LK) cells in order to isolate the virus. Indian sheep (4.05%), goats (7.25%) and cattle (9.15%) showed serum antibody titers against the adenovirus. Different cell cultures of sheep and goat origin supported the growth of the adenovirus but there were some differences in showing CPE and virus yield. Lung cell cultures showed a later CPE than kidney and testicular cultures, whereas the kidney cell cultures yielded higher titers than did testicular and lung cell cultures.

Belák and Rusvai studied the occurrence of natural in utero adenoviral infection in sheep. They isolated three adenoviral strains from the kidney of 174 sheep fetuses and showed their relationship to BAV 2. Neutralizing antibodies against BAV 2 were present in 20% (5/25) blood samples of sheep fetuses. This study proved that transplacental transfer of adenovirus infection is possible naturally.

Goyal and colleagues conducted a study for seroprevalence of antibodies to seven viruses in Minnesota (USA) in healthy ewes. The researchers discovered that 7.6% of the ewes tested vectored BAV 3.

LeaMaster and coworkers performed an epizootologic study of respiratory tract diseases and isolated OAV 5 and 6 in recently weaned lambs. Both serotypes were found jointly infecting the lambs, which showed signs of respiratory tract disease. The morbidity rate was 13% whereas the total mortality was 4.1%; 57.6% of which was due to pneumonia. Adenovirus was present in 36% of lung specimens with pneumonia.
Equine Adenovirus

Few articles concerning equine adenoviruses (EAV) have been published. However, EAV infection is not a major problem in horses. These viruses are generally pathogens of low virulence in normal healthy horses. When clinical disease occurs, it is usually mild and limited to the upper respiratory system. Only in immuno-compromised individuals do adenoviruses regularly cause severe clinical disease. So far, the majority of isolations have proven those viruses to be of a single serospecies designated equine adenovirus species 1. This adenovirus is reportedly present worldwide and is apparently species specific.22

A team of Japanese researchers recovered EAV from racehorses in Japan. These horses had been showing chronic pyrexia for 6 years. However, the team was unable to conclude that the clinical sign was due to EAV since there were also some other viruses found in the same horses.109

Llama Adenovirus

Not much is known about llama adenoviruses, for llama is a newly studied animal species. The work toward the investigation of viruses in llama has been started at Oregon State University. Mattson and coworkers4 have recently identified an adenovirus in llama with diarrhea and designated it 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.76

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III. MATERIALS AND METHODS

Media

Medium used for growth and maintenance of cell cultures and for dilution of virus and serum was Minimal Essential Medium (MEM) with Earles salts. 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

Primary bovine kidney (BK) and bovine spleen (BS) cell cultures were propagated from a 6-month-old bovine fetus. Llama kidney (LLK) cell cultures were propagated from a 4-month-old llama. Primary cell cultures were prepared by the trypsin digestion method. Bovine turbinate (BT) cell and ovine fetal corneal cells (OFC) were received from the National Animal Disease Service Laboratory. Stock cultures of primary cells and cell lines were transferred with a 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 microtitre plates.

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

c National Veterinary Service Laboratories. PO Box 844, Ames, Iowa 50010.
Virus Source

Bovine adenovirus (BAV) species 1-2 and 4-8 were purchased. Ovine adenovirus species 5, strain RTS 42 (OAV 5) and species 6, strain RTS 151 (OAV 6) were received from Dr. Howard Lehmkuhl. Ovine adenovirus strain OAV 47F and OAV 475N were isolated in Oregon from lambs. Equine adenovirus species 1 (EAV 1) was received from the National Animal Disease Laboratory. Bovine adenovirus species 3 strain 5C was isolated in Oregon from the conjunctiva of a calf with conjunctivitis and diarrhea. Bovine adenovirus strain 32CN (BAV 32CN) was isolated from a 4-month-old calf with conjunctivitis. Llama adenovirus strain 7649 (LAV 7649) was isolated from the feces of a 2-week-old cria with diarrhea.

Stock virus was allowed to replicate in the following cell cultures (Table 1):

Table 1. List of cell cultures used for replicating bovine, ovine, llama, and equine adenoviruses.

<table>
<thead>
<tr>
<th>Type of cell cultures</th>
<th>Adenovirus species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine kidney (BK) cell culture</td>
<td>BAV 1 and BAV 2</td>
</tr>
<tr>
<td>Bovine turbinate (BT) cell culture</td>
<td>BAV 3 and BAV 32CN</td>
</tr>
<tr>
<td>Bovine spleen (BS) cell culture</td>
<td>BAV 4, BAV 5, BAV 6, BAV 7, BAV 8,</td>
</tr>
<tr>
<td>Ovine fetal corneal (OFC) cell culture</td>
<td>OAV 5, OAV 6, OAV 47F, and OAV</td>
</tr>
<tr>
<td>Llama kidney (LLK) cell culture</td>
<td>LAV 7649</td>
</tr>
</tbody>
</table>

Virus (10 ml suspension) was inoculated upon cell culture monolayers in 490 cm² 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.

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d American Type Culture Collection, 12301 Parklawn Dr. Rockville, MD 20850.
e Howard Lehmkuhl, DVM, PhD., National Animal Disease Laboratory, Ames Iowa 50010.
f Donald Mattson, DVM, PhD., Personal Communication. College of Veterinary Medicine Oregon State University, Corvallis, OR 97331.
g Idem footnote f.
after cytopathic effect (CPE) involved nearly 100 percent of cells (usually 7 to 12 days). Cells were scraped 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.\textsuperscript{h} Titer of the virus was calculated by the Reed-Muench formula.\textsuperscript{71}

**Antiserum Production**

Virus replication for production of antiserum in rabbits was similar to that which has been described. When culture flasks were ready to harvest, the cells were scraped from the flask. The infected cells were centrifuged 3000 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 trichlorotrifluoroethane (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 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.

\textsuperscript{h} Catalogue No. 25861, Corning Glass Works. Corning, New York 14831.
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 (4 sites) and intramuscularly (1 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.

**Virus Neutralization Test**

Antigenic distinctiveness among the different adenoviruses was determined by the virus neutralization (VN) test. Antisera to the viruses were thawed and diluted in 2-fold steps in 50 μl MEM in flat bottom microtiter plates. 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 1:16. Plates were incubated at 37 C for 60 minutes after which 50 μl of MEM containing 5 x 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 to 8 days. Serum end point were determined by the Reed-Muench formula.
IV. RESULT

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 2).

Table 2. Reciprocal virus neutralization of the bovine, ovine, llama, and equine adenoviruses.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Antiserum against</th>
<th>BAV 1</th>
<th>BAV 2</th>
<th>BAV 3</th>
<th>BAV 4</th>
<th>BAV 5</th>
<th>BAV 6</th>
<th>BAV 7</th>
<th>BAV 8</th>
<th>32CN</th>
<th>OAV 5</th>
<th>OAV 6</th>
<th>OAV 47F</th>
<th>OAV 475N</th>
<th>LAV 7649</th>
<th>EAV 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAV</td>
<td>BAV</td>
<td>6,183</td>
<td>600</td>
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<td></td>
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<td></td>
<td></td>
<td>353</td>
<td>1,304</td>
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<tr>
<td>BAV 2</td>
<td></td>
<td>32</td>
<td>118,696</td>
<td>27</td>
<td>3,049</td>
<td>1,557</td>
<td>69</td>
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<td>BAV 3</td>
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<td>3,098</td>
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<td>BAV 4</td>
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<td>508</td>
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<td>58</td>
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<td>BAV 6</td>
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<td>6,080</td>
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<td>32CN</td>
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<td>193,065</td>
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<td>1,951</td>
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<td>76</td>
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<td></td>
<td></td>
<td>19,100</td>
<td>6,432</td>
<td>87</td>
<td>22</td>
<td>44,700</td>
<td>7,461</td>
<td>48</td>
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<tr>
<td>OAV 475N</td>
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<td>LAV 7649</td>
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<td>EAV 1</td>
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<td>65,015</td>
</tr>
</tbody>
</table>

The numbers indicate reciprocal of the serum dilution. Titer that is equal or higher than 1:22 is regarded positive. Highlighted cells are homologous tests. Blank cells are negative, i.e., titer < 1:22.
Bovine Adenoviruses

BAV 1, which belongs to the subgroup 1, was neutralized not only by its homologous antiserum (1:6,183) and BAV 2 (1:600), but also by OAV 6 (1:1,304) and by the antiserum to the bovine adenovirus isolate 32CN (1:353). However, the neutralizations of BAV 1 and BAV 2 viruses by both of the OAV 6 and 32CN antiserums were one way. On the other hand, antiserum to BAV 1 one-sidedly neutralized the OAV 47F virus, while its neutralization to BAV 2 virus (1:32) was considerably weaker than the reciprocal (1:600).

BAV 2, a member of subgroup 1, was neutralized by its homologous antiserum (1:118,696) and by BAV 1 antiserum (1:32). This virus was also strongly neutralized by OAV 5 antiserum (1:3,049) and OAV 47F antiserum (1:1,557). The pattern of the neutralization by these antiserums was two-sided. The BAV 2 was also neutralized by 32CN and llama adenovirus (LAV) antiserums, 1:27 and 1:69, respectively. Nevertheless, these neutralizations were weak and only one way.

Another member of BAV subgroup 1, BAV 3, was found not to cross-react with the other viruses. This virus was only neutralized by its own antiserum (1:3,096).

Bovine adenovirus species 4 and BAV 8, members of subgroup 2, had a 1:508 and 1:2874 end point by their own homologous antiserums, respectively. These viruses were also found to have a two-way cross-neutralization to each other. The BAV 4 was neutralized by BAV 8 antiserum (1:58). Likewise, the antiserum to BAV 4 neutralized the BAV 8 (1:22). The BAV 8 also two-sidedly interacted with BAV 5 and this antigenic interaction was rather weak. The antigenic relationship was 1:75 for both ways.
The BAV 5 was neutralized by its own homologous antiserum with a titer of 1:2400. Besides being neutralized by BAV 8 antiserum, it was also partially neutralized by OAV 475N. However, this neutralization was one-sided and the titration end point for this reaction was 1:32.

Results of this study showed that BAV 6 and BAV 7 were merely solitaires. They did not cross-react with any of the other viruses. The homologous antiserum titrations for these viruses were 1:6080 and 1:6770, respectively.

**Bovine Adenovirus Isolate 32CN and Ovine Adenoviruses**

The 32CN virus was found to be neutralized by many antiserums. However, this virus appeared to have a very close antigenic relationship to the OAV 475N. Antiserum to the OAV 475N neutralized the 32CN at 1:19100 and, reversely, the antiserum to the 32CN neutralized the OAV 475N at 1:44700. There was also a moderately close two way-cross-neutralization between the 32CN and the OAV 6 at 1:508 and reversely at 1:951 end points. Furthermore, antiserum to OAV 475N neutralized OAV 6 at 1:625 in a one-way fashion. The 32CN virus had a two-way cross-reaction with LAV 7649, while the OAV 475N virus was one-sidedly neutralized by LAV 7649 antiserums. But the magnitude of those cross-reactions with LAV 7649 were considerably weak.

**Equine Adenovirus (EAV) Species 1**

This current study was only able to show a very weak antigenic reactivity of the EAV to the 32CN. It was partially neutralized by the 32CN (1:22) and this partial neutralization was one way. The homologous serum neutralization titer for the EAV was 1:65015.
Llama Adenovirus Isolate 7649

It is evident from the data that llama adenovirus isolate 7649 is not closely related antigenically with any of the other viruses. Even though it did possess common antigenic determinants with some other adenoviruses, i.e., with 32CN (1:38 vs. 1:87) and OAV 5 (1:32 vs 1:24), the degree of the relatednesses were only minor. The antiserum to LAV isolate 7649 did neutralize the BAV 2, OAV 47F, and the OAV 475N at low level and in one-way fashion. The homologous titer of the LAV isolate 7649 was 1:172,674.
V. 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.$^{119}$

Using the above parameters, this investigation has shown that ovine adenovirus species 5, strain RTS-42 is of the same antigenic species as Oregon isolate OAV 47F, a 1:1 and 1:1 test and reciprocal antibody ratio (TRAR) was shown. Likewise, Oregon isolate OAV 475N is of the same antigenic species as Oregon isolate 32CN with a 1:1 and 1:4.3 TRAR. Since all ovine adenoviruses were not available for comparison, it is not known if the 32CN - OAV 475N virus isolates are of a previously unrecognized species or if they can be classified as one of the recognized OAV species 1-4.

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 culture derived from the natural host.$^{59,99}$ 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 genera. 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.99

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 is the first study where the antigenic nature and cell culture specificity of a llama adenovirus has been studied. This study represents the first time an adenovirus from llamas has been characterized antigenically. While additional adenovirus from this species of animal 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 antigenic components. 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.20 This research has shown that bovines and ovines appear to be able to be infected with isolate 32CN (a bovine isolate). Further, previous studies conducted by others have shown that sheep can be infected with BAV species 2 and species 7.3,14

The antigenic composition of human adenoviruses has been studied more extensively than any other species. Several human adenoviruses share common antigenic determinants eventhough they are classified as different species. It is not
uncommon to have several members of the 41 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).\textsuperscript{118} This observation has 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, it would be prudent to use a species of virus with a broad heterotypic antigenic profile for the species of animal in question, e.g., for cattle BAV species 8 and for sheep strain 32CN/OAV 475N.
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