The characterization of two bovine adenoviruses isolated from calves exhibiting clinical and pathological signs of weak calf syndrome
by Patricia Knox Shadoan

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Veterinary Science
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Characterization of isolates 18115 and 1-1504 involved the determination of their nucleic acid type, determination of size and shape, presence of essential lipid, pH and heat sensitivity, growth properties, and serologic analysis.

Infectivity of the, viruses was inhibited by the thymidine analogue, IUDR, and staining of virus-infected cell cultures with acridine orange demonstrated the bright yellow-green fluorescence characteristically produced by DNA-containing viruses.

Lack of essential lipids was ascertained by the resistance of the isolates to inactivation by the lipid solvent, chloroform. Little or no susceptibility to either heat or acid conditions (pH 3.0) was demonstrated.

By the process of ultrafiltration and transmission electron microscopic examination, the viruses were shown to be spherical particles approximately. 70 nm in diameter.

Isolate 18115 and 1-1504 produced cytopathic effect in primary and low passage bovine lung, salivary gland, turbinate, testicle and thyroid cell cultures, but failed to effect pathologic change in primary bovine kidney and an established bovine kidney (GBK) cell line. The inclusions generated by the viruses were multiple nuclear bodies that were regular, often round, in shape.

Isolates 18115 and 1-1504 were neutralized by antiserum produced against serotype 7 bovine adenovirus and positive fluorescence was observed by the indirect fluorescent antibody test in virus-infected cell monolayers treated with antiserum to bovine adenovirus serotype 7.

Bovine adenoviruses are known to be important in the etiology of bovine respiratory and enteric disease; the isolation of these agents from the tissues of weak calves indicates that additionally they may be of etiologic importance in the pathogenesis of weak calf syndrome.

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Date March 23, 1979

THE CHARACTERIZATION OF TWO BOVINE ADENOVIRUSES ISOLATED FROM CALVES EXHIBITING CLINICAL AND PATHOLOGICAL SIGNS OF WEAK CALF SYNDROME

by

PATRICIA KNOX SHADOAN

A thesis submitted in partial fulfillment
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Approved:

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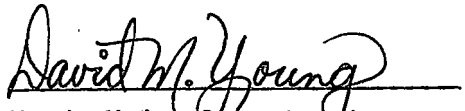

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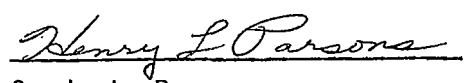

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ABSTRACT

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CHAPTER 1

INTRODUCTION

Weak Calf Syndrome

Weak calf syndrome (WCS) is a disease entity of newborn calves that was first recognized in the Bitterroot Valley of western Montana in 1964. Four years later it was seen in Idaho, where veterinary practitioners diagnosed 400 cases around the Salmon-Challis area. Since that time, the condition has been frequently observed in Beaverhead County, Montana and Custer County, Idaho, and has also been seen sporadically in other areas of these two states. Additionally, it has been reported in several other states including California, Colorado, Nevada, Oregon and Nebraska. Calf losses during spring calving of between 6 to 15 percent and up to as many as 50 percent in individual herds implicate it as the cause of great economic loss. This disease entity is known by a variety of terms including neonatal idiopathic polyarthritis, Ward's syndrome and Bitterroot crud, but is most frequently referred to as WCS (12, 25, 31).

Clinical signs attributed to this disease develop within the first ten days of the calf's life, the majority of the afflicted calves showing signs of illness between three and seven days of age. Among the usual presentation of clinical signs accorded this syndrome are severe debilitation and depression; the calf is weak and unable to stand and nurse. Often the animal's muzzle is encrusted and bright

red in color. Many calves are lame and show an inability or unwillingness to stand, or may stand with an arched back and be reluctant to move. Front and rear legs may exhibit edema and swelling of the periarticular tissues of the carpel and tarsal joint sacs. The conjunctiva and third eyelid may show petechial and diffuse hemorrhage. The body temperature is usually normal, dropping below normal as death approaches. Diarrhea and dehydration are not initially observed but are usual sequelae if the calf lives for several days. Mortality rates vary from 60 to 80 percent in untreated cases (12, 25, 31).

Of the lesions noted during necropsy, the most outstanding are subcutaneous hemorrhages and edema in the tissues of the extremities. These are noticeable primarily around the hock and knee joint, and extend distally over the lateral aspects of the respective limbs. The edema is most pronounced around the hock joint and tissues supporting the Achilles tendon. These particular lesions are the ones evident most commonly in calves with WCS and are seen in 95 to 100 percent of the cases.

The second most striking gross lesion is a polyarthritis with hemorrhagic synovial fluid containing varying amounts of fibrin. Of 25 instances of WCS from the Salmon-Challis area of Idaho, this was present in 65 percent of the cases.

In approximately 30 percent of the cases, lesions of the forestomach and abomasum are seen involving diffuse congestion and hemorrhage, with occasional vesiculation, erosion, and ulceration of the epithelial lining. In severe cases, hemorrhage and edema can be observed on the ventral aspect of the thorax and neck (12, 25, 31).

Factors thought to contribute to the severity of clinical illness in WCS outbreaks include the stress of inclement weather and the quality of herd management (12, 25). Studies conducted by Bull and co-workers indicate nutrition levels may also be an important consideration (10).

Attempts to recover infectious agents from weak calves have met with varied results. In 1969 Page and associates isolated a previously undescribed mycoplasmal agent from the placenta of a cow that gave birth to a weak calf (58) and a bacterial agent, Hemophilus somnus, was recovered by Waldham from the vaginal mucous of a dam that produced a weak calf (76). Neither of these organisms have been shown to be the causative agent of WCS, although trials with Hemophilus somnus produced a polyarthritis similar to that found in field cases (76).

Several viral agents have been isolated from tissues of weak calves over the last several years by investigators in Montana, Idaho, and Iowa. Coria from the National Animal Disease Center (NADC) in Iowa, and Stauber at Idaho State University both isolated bovine viral

diarrhea (BVD) virus and an adenovirus (AV) from weak calf tissues. Characterization studies show these AV isolates to be serotype 7 bovine AV (BAV) (15, 73).

Cutlip and McClurkin from NADC have reported results of their experimental infections of young calves with BAV-7. They were able to reproduce hemorrhagic and necrotic lesions of the subcutaneous tissues of the legs and joints that are commonly associated with WCS, but they also observed hemorrhagic and necrotic lesions of internal organs (kidneys, adrenal gland, and liver) that are not generally noted (17, 45).

Experimental intraamnionic exposure of bovine fetuses to BAV-7 produced calves that were born showing clinical signs resembling those described for WCS. However, the gross and microscopic lesions generated in this experiment were distinctly different from those described for the syndrome and it was concluded that the disease produced was not WCS (72).

Statement of Purpose

During the 1975 calving season, Dierks, Smith, and Gollehon of Montana State University, isolated 22 viral agents from the tissues of 10 suspected weak calves, fetuses, and one lamb (26). The purpose of the work reported here was to characterize, by studies determining the

physicochemical and biological properties, two of the virus isolates recovered from the tissues of these weak calves.

CHAPTER 2

LITERATURE REVIEW

Adenoviruses

In 1953, Rowe and associates isolated a cytopathic agent from human adenoid tissue that they had been culturing as a potentially favorable host for an elusive respiratory virus. After a prolonged incubation period, pathologic changes were noted in uninoculated as well as inoculated cell cultures. This cytopathic response was shown to be due to the emergence of a previously unidentified virus from latent infections of the adenoid tissue (69). The designation "adenovirus" was officially given to this group of viruses to indicate their original isolation site (28).

AV are widespread in nature and now comprise a well-defined group of viruses. Members of this family are simple viruses composed of a core of double-stranded DNA and a surrounding protein coat arranged in the form of an icosahedron. Two hundred fifty-two capsomeres comprise this outer protein shell of which 240 are hexon capsomeres and 12 are penton capsomeres. The pentons are apical subunits upon which are found fibers responsible for attachment to the host cell (24).

Mature AV particles range between 65-90 nm in diameter, averaging about 70 nm in diameter. These viruses are non-enveloped and subsequently exhibit resistance to lipid solvents

(i.e., chloroform or ether) (59). They are acid stable, but are subject to inactivation at 56^{o} C for 10 minutes (63).

AV multiplication and maturation takes place in the nucleus of susceptible cells, producing characteristic cytopathic effect (CPE) and type B intranuclear inclusions. The "cell detaching factor" or "toxin" that is a property of the intact penton is responsible for the typical rounding, clumping and detachment from surfaces that is seen in infected cells (24).

The antigenic character of the AV depends largely on its morphological subunits. The hexon capsomere contains the group-specific or complement-fixing (CF) antigen as well as a type-specific reactive site. The major type-specific antigen as well as minor subgroup antigens are found in the purified fiber, the length of which varies according to serotype. The penton provides some minor antigens of the virion and is also found as a family-reactive soluble antigen (24, 59, 83).

This family of viruses is highly species specific and is arbitrarily divided, according to the natural hosts, into six subgroups represented by human, simian, bovine, canine, murine and avian AV. All except the avian types share a common CF antigen (59).

Bovine Adenoviruses

The first isolations of AV from the bovine species were made in the United States by Klein and co-workers (39). As the result of their search for viruses responsible for the production of poliovirus antibodies in cattle, two AV were isolated in bovine kidney cultures from the feces of clinically normal cattle. These two virus strains, designated bovine no. 10 and bovine no. 19, are now considered the prototypes representing BAV type 1 and type 2, respectively. Neutralization of these bovine viruses by human gammaglobulin shows the possibility of a relationship to human AV (39, 40). Subsequently, BAV-3 was isolated in bovine kidney cultures from the conjunctiva of an apparently healthy cow in Britain (20).

Pathogenesis studies of experimental infection of calves with these BAV have produced varied results. Klein recorded that the intranasal inoculation of two calves with BAV-1 (bovine no. 10) effected a neutralizing antibody response but no concommitant symptoms (40). Using strain 10088 of BAV-1, Mohanty and Lillie demonstrated the production of marked clinical signs of respiratory distress and diarrhea when this virus was inoculated intranasally and and intratracheally into 6 to 12-week-old calves. Calves infected via the ocular route displayed no noticeable response (56).

Strain WBR 50 of BAV-1, recovered by Darbyshire from the nasal swabs of a calf suffering from pneumonia, induced type-specific

hemagglutination-inhibiting antibodies and mild clinical signs in experimentally infected calves (18).

In other pathogenesis studies conducted by Darbyshire and associates, BAV-3 (strain WBRI) was shown to invoke a mild clinical response characterized by pyrexia, respiratory distress, nasal and conjunctival discharges. Gross pathologic changes were primarily confined to the lungs which showed areas of consolidation, collapse, and emphysema. The histopathologic changes in the lungs consisted of proliferative bronchitis and necrosis with bronchiolar occlusion and associated alveolar collapse. Diarrhea was evident, but not a prominent feature of the disease (20, 21). Pulmonary reactions to BAV-1 (bovine no. 10) and BAV-2 (bovine no. 19) involved gross and microscopic tissue changes similar but less severe than those observed with type 3. These data indicated that type 3 is the most virulent of the three types of BAV (22). Conversly, Ide and co-workers failed to produce signs of respiratory illness in calves they had inoculated with either BAV-3 (WBRI) or a strain of BAV-1 which was isolated in Canada from a non-fatal case of pneumonia (35).

Additional recorded isolations of these viruses have been reported and include that of BAV-1 from a calf with pneumoenteritis (70), from bovine cell cultures (27), and from normal calves. BAV-2 has been recovered as an adventitious contaminant in primary bovine embryonic kidney cell cultures (57) and Mattson describes the

isolation of BAV-3 (strain 5C) from a naturally occuring pneumo-enteritis of newborn calves in Oregon beef herds (50).

All of these BAV, types 1, 2, and 3, have been shown to be associated with field outbreaks of respiratory disease, and neutralizing antibodies to these agents are widely distributed in world cattle populations (9, 17, 19, 21, 30, 43, 50, 55, 68, 84).

In Hungary, three strains of BAV were isolated from calves suffering from diarrhea (4) and 78 strains were recovered from afflicted calves during a severe outbreak of pneumonia associated with enteritis. During recent years this particular condition has been the most widespread viral infectious disease among calves in Hungary, resulting in great economic loss (2). These BAV strains differed serologically from the previously established BAV prototype strains and represented two further types designated serotype 4 (prototype strain THT/62) and serotype 5 (prototype strain B4/65). These viruses were isolated in primary bovine testicle (BT) cell cultures, and failed to grow in any other cell culture (5).

Aldasy and Bartha were able to show experimentally that inoculation with types 4 and 5 produced pneumonia and enteritis in calves (2). In a study conducted by Mohanty, BAV types 4 and 5 caused respiratory illness with pneumonia in infected calves, but did not cause enteritis (55).

Tanaka reported the first recovery of BAV from cattle in Japan. BAV strain Nagano was isolated in primary BT cells from the blood of a 25-month-old bull with pyrexia, soft stools, and reduced appetite. When tested for pathogenicity in calves, this isolate produced generally mild clinical responses including fever, diarrhea, and rhinorrhea. A low-titered viremia was evident in several calves (74).

Studies conducted by Matumoto demonstrated strain Nagano to be serologically related to BAV-4 (52). A conflicting report by Mohanty designates strain Nagano as a new BAV, type 10, unrelated to previously established BAV serotypes (55). More recently BAV-4 isolates have been recovered from Oregon cattle with pyrexia associated with signs of respiratory tract disease (51) and several reports indicate that BAV-4 has been isolated from latent infections of BT cell cultures (6, 61).

In the Netherlands, Rondhuis isolated the prototype strain (671130) of BAV-6 as a latent virus from primary BT cell cultures. As with types 4 and 5, this virus grew only in these cultures. Intratracheal inoculation of type 6 in newborn calves induced mild respiratory illness and catarrhal conjunctivitis. Viremia was associated with the infection, and the virus was found localized in several organs including the CNS (49, 67).

Cole isolated 11 BAV strains in Australia from the lungs of calves with acute pneumonia. On the basis of the two types of CPE produced in monolayers by the isolates, they were divided into two groups designated BIL and RG. The BIL strains were recovered from the lungs of animals with acute exudative pneumonia, and the RG strains from the lungs and/or noses of calves with pneumonia and bronchitis of varying severity. These viruses were isolated only in primary BT cultures and failed to grow in other cell types (13, 14). Strain RG has been serologically identified by Mohanty as being a member of type 6 BAV (55). A mild interstitial pneumonia was produced in calves following intratracheal inoculation with BIL. Here the pathologic reactions were confined to the lung, which evinced areas of hyperemia, consolidation and edema (14).

Wilcox isolated other BAV strains from Australian cattle with conjunctivitis and keratoconjunctivitis. These viruses were isolated in primary BT cultures. Of the two types represented, one strain, KC-6, grew only in primary and secondary BT cultures while the other strain, KC-2, was able to grow in several bovine cell types (81). The KC-2 strain has been identified as a BAV-6 (55).

Another AV closely related to BAV-6 has been isolated from the precapsular lymph node of a cow with leukosis. Experimental inoculation of young calves produced acute rhinitis, undulating fever, swelling of lymph nodes, and in some cases, mild conjunctivitis (54).

A BAV strain was isolated by Inaba and co-workers from cattle in Japan suffering from acute pyrexia accompanied by rhinorrhea and diarrhea (36). This strain, Fukuroi, isolated in primary BT cultures, represents the BAV serotype 7 (36, 52). This BAV serotype has also been reported to have been isolated from newborn calves with weak calf syndrome (15, 26, 73), as well as from latent infections of primary BT cell cultures (61).

BAV type 8, strain Misk/67, was isolated in Hungary from calves with pneumoenteritis. Experimentally, type 8 was non-pathogenic when inoculated into calves (8).

Guenov reported the isolation of strain Sophia-4/67 from cultures of kidney tissue from healthy calves. This isolate showed no serologic relationship in neutralization tests with BAV types 1-8 and Guenov has proposed that this virus represents the new BAV-9 (32). Mohanty has also designated two of the strains isolated in Australia, KC-6 and BIL, as BAV-9 (55). It is not known whether these isolates are of the same serotype as Sophia-4/67, but they cannot be the prototypes for BAV serotype 9, as Guenov's proposal was published prior to that of Mohanty's.

Kretzschmar has reported the isolation of an AV that differs serologically from BAV types 1-9 (42). This may represent either a new BAV serotype 10 or BAV serotype 11, depending upon the classification of isolates BIL and RG.

To date, there are nine recognized BAV serotypes (1). Bartha has proposed the division of these AV into two distinct groups based on differences in their biological and physicochemical properties. He has suggested that strains belonging to serotypes 1, 2, and 3 possess similar properties and should be categorized as subgroup I BAV, and strains belonging to serotypes 4 and 5 as well as the strains designated BIL, Nagano and Fukuroi, should be classified among the subgroup II BAV (3). More recent work includes BAV-9 in the first subgroup (44).

According to Bartha's study, members of subgroup I possess a soluble antigen in common with the human strains of AV, while only traces of the same are detected in BAV of subgroup II. Primary isolations of subgroup I BAV can be made on bovine kidney and testicle cell cultures, generally on the first passage, while subgroup II BAV can only be isolated on bovine testicle and fetal lung cell cultures after several blind passages. Intranuclear inclusions generated by members of the first subgroup are characteristically single and irregular in shape, as opposed to those produced by the members of the second subgroup, which are multiple and regularly shaped. Bartha reports another differentiating criteria to be that of heat sensitivity: Subgroup I BAV are completely inactivated at 56^{o} C after 30 minutes, whereas subgroup II BAV were only reduced in infectivity by such treatment (3). Other studies discounted this as a valid

parameter on the basis that the AV lost their heat sensitivity after several passages in cell culture (7).

All these studies, in addition to serologic surveys of bovine herds which indicate that BAV infection is widespread, have in recent years drawn attention to BAV as important etiologic agents of respiratory and enteric disease.

CHAPTER 3

MATERIALS AND METHODS

Cell Cultures

Several bovine cell types were utilized during this study to ascertain their susceptibility to viral-induced CPE. Primary and low passage kidney, lung, salivary gland, turbinate, testicle, and thyroid cells were obtained from fetuses purchased from the local slaughter house. A bovine kidney (GBK) cell line developed by R. F. Solarzano, Columbia, Missouri, and a BVD-free bovine turbinate (BTU) cell line developed at NADC (46) were also used. The BTU cell line was used almost exclusively for virus propagation and characterization procedures.

The media used for cell cultures was Dulbecco's modified Eagle minimum essential medium (DMEM) supplemented with 10 percent fetal calf serum (FCS) for cell culture growth reduced to two percent FCS for the maintenance of cell culture monolayers. Irradiated bovine serum distributed by Microbiological Associates was used in media for cultures of the BVD-free cell line. All media were buffered with sodium bicarbonate diluted to a final concentration of 0.15 percent. Liquid antibiotics and antimycotics were used for control of contamination and were added to media in the following concentrations: penicillin 100 mcg/ml, streptomycin 100 mcg/ml, and amphotericin B 1-5 mcg/ml. Routinely, cell cultures were grown in 250 ml Costar

plastic tissue culture flasks and incubated at 37^{o} C in a 5 percent CO_2 atmosphere.

The procedures outlined by Younger were used for establishing primary and low passage cell cultures (85). Bovine fetal tissues were taken aseptically and immediately placed in sterile growth medium. After removing superfluous membranes, the tissues were minced with scalpel blades into approximately $1mm^3$ pieces. These fragments were washed three times with serum-free DMEM before being transferred to a 250 ml trypsinizing flask containing 100 ml of a .25 percent trypsin solution. The flask was placed on a magnetic stirrer for 15 minutes, after which time the fluid was decanted and replaced with fresh trypsin. This process was repeated twice more prior to straining the suspension through gauze into a conical centrifuge tube for low speed centrifugation (50-90 xg for 10 minutes). The supernatant fluid was then decanted and the packed cells resuspended in nutrient medium containing antibiotics and antimycotics before being added to flasks in concentrations of between 150,000-500,000 cells/ml.

Cells were subcultured regularly to maintain cell viability and as needed for virus work. To subculture cells, the growth medium was first decanted from flasks and the cell monolayers washed with 5 ml of a calcium and magnesium-free saline, Rinaldini enzyme solution (R-saline) (65). The cells were then covered with 2 ml of a solution of .1 percent trypsin and .04 percent EDTA in R-saline and incubated

for about 10 minutes at 37° C until they detached from the surface of the flask. The cells were dispersed by pipetting several times before being passed into a new flask holding 15 ml of growth medium. Antibiotics and antimycotics were omitted from the media used when subculturing cells.

For stock supplies, concentrated cells were suspended in medium containing 20 percent FCS and 10 percent DMSO by volume, and then sealed into freezing vials. After being brought slowly to -70° C, the vials were immersed in liquid nitrogen for storage until needed.

When infecting cells with virus, newly formed cell monolayers were used whenever possible. When the monolayers were to be infected, the growth medium was removed and the cells washed with R-saline before adding the appropriate viral dilutions. A sufficient amount of virus was added to facilitate an even distribution of virus on the cell sheet. The flasks were then incubated for one to two hours to allow virus adsorption to the cells, after which time the inoculum was removed and maintenance medium added. All manipulations involving cell cultures and inoculation of viruses were conducted under a Bioguard laminar-flow hood.

Virus Titrations

For titrating virus, cell monolayers were established in wells of Linbro plates of 24. The growth medium was decanted from the wells and the cells washed with R-saline. Two-tenths ml of 10-fold virus dilutions made in DMEM without serum was added to the appropriate wells. All titrations were done in quadruplicate. These plates were then incubated for one to two hours at 37^{o} C in plastic containers designed for retaining a humid environment to avoid drying in the wells. The virus inoculum was then pipetted out and replaced with 1 ml of maintenance medium. The plates were checked daily with an inverted microscope for CPE, and the 50 percent tissue culture infective doses ($TCID_{50}$) were calculated by the method of Reed and Muench (64).

Source of Virus

Several viruses were isolated in BTU cell cultures from the tissues of calves showing clinical signs of WCS. Of these, two were selected for characterization studies. Virus isolate 18115 was recovered from the lung tissue of one of the calves after three blind passages in cell culture, and virus isolate 1-1504 was recovered from kidney tissue after four blind passages.

Reference viruses included BAV serotype 3 (strain FS0213) from the Veterinary Medical Research Institute, Ames, Iowa, and BAV

serotype 7, infectious bovine rhinotracheitis (IBR) virus and parainfluenza-3 (PI-3) virus from NADC, Ames, Iowa.

Characterization Studies

The nucleic acid type was established using the DNA inhibitor 5-iodo-2'-deoxyuridine (IUDR) and employing the methods described by Hamparian (33) and Coria (15). Monolayers of BTU cell cultures were grown in DMEM maintenance media with IUDR (100 mcg/ml) for 16-18 hours before inoculation with virus. Similar media was put on the cell cultures after viral adsorption. Determination of nucleic acid type was based on suppression of replication of DNA viruses by IUDR. PI-3 and BAV-7 were used as RNA and DNA controls, respectively.

The method of Feldman and Wang was used to determine virus sensitivity to lipid solvents (29). A mixture of .05 ml analytical reagent grade chloroform and 1 ml tissue culture fluid containing virus was shaken for 10 minutes at room temperature. The mixture was then centrifuged at 60 xg for 10 minutes, and the clear supernatant fluid titrated for infectivity. IBR virus served as the enveloped virus control, and BAV-7 as the non-enveloped control.

Virus particle size was estimated by filtration through a series of filters of graded porosity. Purified virus diluted 100-fold in distilled water was passed through sterile 25 mm nucleopore polycarbonate membranes with pore sizes of 200 nm, 100 nm, 80 nm, and

50 nm. Particle adsorption to filters was reduced by utilizing the methods of Ver (75). The viral content of each filtrate, as well as tissue culture fluid obtained prior to filtration, was assayed.

Additionally, virus size as well as shape was determined by examination of negatively-stained particles with the transmission electron microscope (TEM). In preparation for negative staining, a flask of virus-infected cells was frozen and then thawed a total of three times. The cell culture fluid was centrifuged at 60 xg for 10 minutes and the resulting supernatant fluid was centrifuged at 100,328 xg for one hour. The supernatant fluid was discarded and the remaining material resuspended. A mixture of four drops of the virus suspension, four drops of 2 percent PTA (pH 6.8) and one drop of bovine serum albumin was made, and a drop of this material was put on a formvar-coated 300 mesh grid with a tuberculin syringe. The excess fluid was removed by touching a corner of filter paper to the edge of the grid. After drying, the grid was examined with a Zeiss EM95-2 TEM.

Sensitivity to acid was tested employing the technique established by Ketler (38). Tissue culture fluid containing virus was diluted 1:10 in DMEM adjusted to pH 3.0 or pH 7.0 with McIlvaine's buffer. The mixtures of virus and buffer were held at room temperature for 30 minutes, after which time the residual virus content was determined by titration. Reference strain viruses used

were BAV-7 and PI-3.

Heat sensitivity was tested according to the method of Wallis (77, 78). One ml of virus suspension diluted 10-fold in distilled water was delivered into tubes of uniform size and thickness. The tubes were capped and placed in a 56° C water bath that covered the tubes to within one-fourth inch from the cap. At the end of the 30 minute heating period, the tubes were quickly transferred into an ice water bath, and the samples titered for infectivity. BAV-3 and BAV-7 were used as examples of subgroup I and subgroup II controls, respectively.

Cytopathic Effect and Staining Characteristics

Monolayers of BTU cells were grown on chambered tissue culture slides for examination of viral-induced CPE. Infected and non-infected control monolayers were fixed with Bouin's fixative at various intervals before staining with hematoxylin and eosin (H & E). Slides to be used for acridine orange staining were prepared as directed in the technique developed by Dart (23). For the indirect fluorescent antibody test (FAT) the methods of Potgieter were followed (62). The conjugate used for immunofluorescent staining was fluorescein isothiocyanate (FITC), conjugated antibovine antibody of rabbit origin (Miles Laboratory). This was used in dilutions of 1:16.

These stained and mounted preparations were examined and photographed with a binocular Leitz fluorescent microscope equipped with a standard light source. Standard light microscopy was utilized for the H & E stained slides and ultraviolet light for examining acridine orange and indirect FAT preparations.

Serum-virus Neutralization Test

Serum-virus neutralization tests were performed in microtiter plates using standard procedures. Serial two-fold dilutions of the serum were made in microtiter transfer plates. To this was added an equal volume (.05 ml) of 50 $TCID_{50}$ of test virus. The serum-virus mixtures were incubated for one to two hours at 37^{o} C before being transferred to microtiter plates containing monolayers of the appropriate cells. The plates were maintained in a CO_2 incubator at 37^{o} C until examination with an inverted microscope.

CHAPTER 4

RESULTS

Nucleic Acid Type

As shown in Table 1, treatment of isolates 18115 and 1-1504 with IUDR resulted in a two to three log unit decrease in the titer of these viruses. This sensitivity is presumptive evidence that these are DNA-containing viruses. The RNA virus control, PI-3, was unaffected by similar treatment while the DNA virus control, BAV-7, was reduced in titer.

Table 1. Effect of IUDR on Virus Titer

Virus	Titer of Control[a]	Titer after IUDR Treatment[a]	Nucleic Acid Type
18115	5.00	2.20	DNA
1-1504	4.20	2.20	DNA
BAV-7	3.00	1.20	DNA
PI-3	7.20	7.70	RNA

[a]Titers expressed as $\log_{10}$ $TCID_{50}$ per/ml.

Further evidence supporting these results was provided by the acridine orange staining of virus infected cell monolayers. Both