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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 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 2-9. Digestion with the *Pst*I enzyme produced more RE bands than others, while *Bam*HI yielded fewer bands. Enzyme *Pst*I cleaved all the viral strains (13/13) into different number and size of bands while *Eco*RI did not digested all the strains (8/13). Bands cleaved by the different enzymes varied in molecular weight from 0.88 Kbp to 24.9 Kbp. 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 groups. 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 mention the number or size of RE bands produced by different restriction enzymes, (Pommer, 1991). There are several reports regarding this technique in the literature for human, (Adrian, 1986), bovine, (Benkö, 1988) and porcine adenoviruses, (Kleiboeker, 1993). 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.

Rowe and coworkers first isolated a cytopathogenic agent from human adenoids and proposed the term Adenoid Degeneration Agent (Rowe, 1953). These agents were isolated from patients with acute respiratory illness and termed Respiratory Illness Agent, (Hilleman, 1954). Similarly, other group proposed the term Adeno Pharyngeal Conjunctival agents as the group name (Huebner, 1954). After two years another group of researchers proposed adenoviruses as the group name for these viruses (Enders, 1956). 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 that consist of Mastadenovirus (adenovirus of mammals) and Aviadenovirus (adenovirus of avians) (Fenner, 1975).

The characteristics of adenovirus have been defined by many workers (Ginsberg, 1962), (Huebner, 1954) and (Willner, 1969). Adenoviruses from all species are nonenveloped, icosahedral, 700-800 A 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), (Fraenkel-Conrat, 1974). Associated with their genome there is a 55K protein, which plays a role in replication, (Fenner, 1993) and also gives a unique advantage in separation of viral genome from the fragmented cellular DNA of infected cell (Shinagawa, 1983). 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) that carry fibers; a fiber on each penton (Ginsberg, 1966). The hexons are polygonal prisms with a central hole (Wilcox, 1963). 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 (Norby, 1969). Adenoviruses are resistant to the action of lipid solvents, such as ether (Huebner, 1954), chloroform (Feldman, 1961) fluorocarbons (Wilcox, 1963) and deoxycholate (Horsfall, 1965) indicating that the viruses lack essential lipid. Adenoviruses are acid stable (Hamparian, 1963), (with few exception i,e., few strains are sensitive to pH 3) and, in fact, are more stable at acid than alkaline pH (Wilcox, 1963). They are also relatively stable in homogenates of infected cells (Duby, 1986).

Several different procedures are described in the literature for the isolation of viral DNA from infected cells. Originally the technique to purify adenoviral DNA from KB monolayer, was described by (Green, 1964). Subsequently, additional modifications in the original procedures were made (Hirt, 1967) & (Pignatti, 1979). Presently two procedures are common for the purification of viral DNA from infected cells. The first procedure is simply to cultivate the virus in appropriate cell cultures and then lyse the infected cells with sodium dodecyl sulfate (SDS), phenol extraction of proteins, and precipitation of 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 (Shinagawa, 1983) as it requires the additional work of labeling 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 endonucleases are those that catalyze cleavages within a nucleic acid strand. Among endonucleases there is a special class of enzymes, restriction enzymes (RE), that recognizes a certain number of nucleotides and cleaves the DNA strand at specific sequences. 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 (Mathews, 1990). The following lists 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

Strains of Ovine Adenovirus:

Six prototypes of ovine adenovirus (OAV) (Adrian, 1986) 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 (Yousaf, 1993). Isolate 32CN was isolated from a bovine with conjunctivitis and respiratory disease (Yousaf, 1993) Llama adenovirus isolate 7649 was isolated from feces of a cria while isolate LA5330 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, 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 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 it 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 for 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 the restriction enzyme's buffers, specific to each RE (provided by the supplier), digestion was carried out in a 20µl volume (quantity of DNA in each sample was higher than 5µg), which also included Ribonuclease A (1 µl of 100µg/ml). Digested samples were incubated at 37°C for 90 minutes. Digestion process was stopped by adding 5µl 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). Negatives from polaroid pictures were developed for scanning. Standard molecular weight markers were used to determine size of RE fragments.

Gel Scanning:

Polaroid picture's negatives were used for scanning by using a scanner. This procedure marked the migration distance of each band and also gave the density of bands. Scanner software (provided with scanner) made a standard curve for each agarose gel on the basis of migration distance of standard molecular weight markers that helped in calculating molecular weight of RE bands for different strains. Migration distance marked by scanner for each RE band was also confirmed by visual/manual measurements.

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 ≥ 0.42 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 DNA samples produced different number and sizes of bands when digested with the four different restriction enzymes (Table 1). All four RE digested both llama adenovirus isolates (Fig 1) and yielded different numbers and sizes of RE bands. Restriction enzyme *Pst* I produced more fragments as it cleaved all the adenoviral DNA samples 13/13 (Table 2, Fig 2), while *Bam* HI cut 12/13 samples (Table 4, Fig 4), RE *Hind* III cleaved 10/13 (Table 5, Fig 5) and *Eco* RI digested the least number of samples 8/13 (Table 3, Fig 3). When all

bands for each strain produced by these enzymes were estimated, to range in size from 0.88 Kb to 24.9 Kbp. A method file (for each gel) was created in order to calculate molecular weight of RE bands by using the migration distance and known molecular weight markers. The gel scanner marked the migration distance of each band and also calculated its molecular weight, in relation to migration distance of molecular weight markers. Average molecular weight of each strain, as digested with these four enzymes ranged between 31.31 Kbp to 33.39 Kbp (Table 6).

Table 1. Number of bands and estimated weight of different ovine and llama adenoviruses after digesting with four different restriction enzymes.

S#	Sample Type	*Bam* HI		*Hind* III		*Pst* I		*Eco* RI	
		B #	M Wt; Kbp	B #	M Wt; Kbp	B#	M Wt; Kbp	B #	M Wt; Kbp
01	OAV-1	4	34.31	9	31.46	6	31.73	2	32.00
02	OAV-2	2	32.28	8	32.49	9	32.15	4	31.64
03	OAV-3	2	33.30	9	31.04	6	32.68	3	30.64
04	OAV-4	2	32.79	9	31.99	5	31.35	5	31.32
05	OAV-5	4	35.25	5	33.65	5	31.98	2	32.60
06	RTS-42	3	33.65	7	32.22	7	31.85	UC	-
07	OAV-6	4	33.20	UC	-	9	30.88	UC	-
08	RTS-151	4	32.72	3	33.43	7	31.60	UC	-
09	475-N	4	31.89	UC	-	9	31.71	UC	-
10	32-CN	4	32.62	UC	-	7	32.57	UC	-
11	47F	UC	-	8	32.45	8	32.24	2	31.90
12	LA-7649	2	31.57	5	31.02	5	31.32	7	31.32
13	LA-5330	2	31.71	4	31.22	5	31.98	5	31.79
14	OFC	-	-	-	-	-	-	-	-
15	LMK-1	-	-	-	-	-	-	-	-
16	MM-9780	6	-	6	-	6	-	6	-
17	MM-0672	5	-	5	-	5	-	5	-

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

Table2. Number of bands and their estimated weight of ovine and llama adenoviruses digested by enzyme *Pst* I.

S#	Sample Type	B #	Kbp								
			#1	#2	#3	#4	#5	#6	#7	#8	#9	Total
01	OAV-1	6	9.00	7.43	6.59	4.16	3.53	1.02				31.73
02	OAV-2	9	12.2	5.11	3.58	3.01	2.25	1.76	1.71	1.47	1.06	32.15
03	OAV-3	6	13.5	6.14	5.20	4.28	1.99	1.57				32.68
04	OAV-4	5	13.5	7.53	6.61	2.64	1.07					31.55
05	OAV-5	5	10.5	8.55	5.60	4.49	3.84					32.98
06	RTS-42	7	10.7	6.14	4.52	3.82	2.94	2.08	1.65			31.85
07	OAV-6	9	6.59	5.67	5.23	3.61	2.95	2.48	1.87	1.48	1.00	30.88
08	RTS-151	7	9.34	6.57	5.01	3.55	2.71	2.52	1.90			31.60
09	475-N	9	6.75	5.35	4.62	4.14	3.43	2.97	1.91	1.51	1.03	31.71
10	32-CN	7	9.53	7.79	4.63	4.00	3.45	2.14	1.03			32.57
11	47-F	8	8.40	6.10	4.53	3.88	3.39	2.94	1.95	1.05		32.24
12	LA-7649	5	14.6	7.85	4.08	3.62	1.17					31.32
13	LA-5330	5	15.9	7.05	4.15	3.44	1.37					31.98
14	OFC	-										
15	LMK-1	-										
16	MM-9780	6	23.1	9.41	6.55	4.36	2.32	2.02				
17	MM-0672	5	1.35	1.07	0.87	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

Among the RE used, enzyme *Pst* I appeared to be most useful as it cleaved all adenovirus strains (13/13, Table.1). The number of bands produced by this RE ranged between 5-9 (Table 2), i.e., there were three to eight cleavage sites in all adenoviruses as recognized by this RE. Molecular weight of different RE bands produced by this RE ranged from 15.9 Kbp (for strains having four cleavage sites) to 1.00 Kbp (for strains having nine cleavage sites). It should be noted that, as the number of restriction sites increased, molecular weight of bands decreased but combined weight of all the bands remained within the average molecular weight of adenoviruses. Restriction enzyme *Pst* I did not produced any visible band when used to digest DNA of uninfected OFC (Fig.2 and LMK-1 cells (Fig.).

Restriction enzyme *Bam* HI digested all (12/13) adenovirus strains except isolate 47F (Table 4). The number of band generated by this enzyme ranged between 2-4 i.e., have only one to three restriction sites. As this enzyme produced low number of bands, molecular weight of different bands was higher than the bands produced by other restriction enzymes. Molecular weight of bands ranged between 27.9 Kbp (for one cleavage site) to 2.03

Kbp (for three cleavage sites). Uninfected OFC (Fig 3.3) and LMK-1 (Fig 1) did not produce 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 5). Number of bands produced by this RE ranged between 3-9 i,e., have two to eight cleavage sites. As this RE has more frequent restriction sites, it produced lighter bands than other RE used. The molecular weight of each band ranged between 24.9 Kbp (for two restriction sites) to 0.88 Kbp (for eight restriction sites). Restriction enzyme *Hind* III did not produced any visible band when used to digest DNA of uninfected OFC (Fig 4) and LMK-1 cells (Fig 1).

Restriction enzyme *Eco* RI appeared to be less successful than other RE as it cleaved less adenovirus strains (8/13) than others (Table 3). It yielded two to seven bands i,e., have only one to six restriction sites. The molecular weight of bands ranged between 17.9 Kbp (for one restriction site) to 1.82 Kbp (for six cleavage sites). This RE did not produce any visible band when used to digest DNA of uninfected OFC (Fig 5) and LMK-1 cells (Fig 1).

Table 3. Number of bands and their estimated weight of ovine and llama adenoviruses digested by restriction enzyme *Eco* RI.

S#	Sample Type	.....Kbp.....								
		B #	B #1	B #2	B #3	B #4	B #5	B #6	B #7	Total
01	OAV-1	2	16.8	15.2						32.00
02	OAV-2	4	15.8	9.00	4.27	2.57				31.64
03	OAV-3	3	16.5	9.18	4.96					30.64
04	OAV-4	5	16.2	5.71	4.25	3.93	1.23			31.32
05	OAV-5	2	20.7	11.9						32.60
06	RTS-42	UC								-
07	OAV-6	UC								-
08	RTS-151	UC								-
09	475-N	UC								-
10	32-CN	UC								-
11	47-F	2	17.9	14.0						31.90
12	LA-7649	7	9.24	6.29	4.34	4.00	3.18	2.45	1.82	31.32
13	LA-5330	5	14.4	6.31	4.64	3.45	2.99			31.79
14	OFC	-								
15	LMK-1	-								
16	MM-9780	6	23.1	9.41	6.55	4.36	2.32	2.02		
17	MM-0672	5	1.35	1.07	0.87	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

Table 4. Number of bands and their estimated weights of ovine and llama adenoviruses digested by restriction enzyme *Bam* HI.

S#	Sample Type	.....Kbp.....					
		B #	B #1	B #2	B #3	B #4	Total
01	OAV-1	4	22.10	6.59	3.59	2.03	34.31
02	OAV-2	2	22.90	9.38			32.28
03	OAV-3	2	23.30	10.0			33.30
04	OAV-4	2	23.50	9.29			32.79
05	OAV-5	4	22.50	6.81	3.85	2.09	35.25
06	RTS-42	3	24.30	7.05	2.30		33.65
07	OAV-6	4	20.06	7.09	3.24	2.27	33.20
08	RTS-151	4	20.00	7.07	3.35	2.30	32.72
09	475-N	4	19.10	7.11	3.37	2.31	31.89
10	32-CN	4	19.80	7.13	3.41	2.28	32.62
11	47-F	UC					-
12	LA-7649	2	27.90	3.67			31.57
13	LA-5330	3	23.00	6.47	2.24		31.71
14	OFC	-					-
15	LMK-1	-					-
16	MM-9780	6	23.13	9.41	6.55	4.36	-
17	MM-0672	5	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

Table 5. Number of bands and their estimated weight of ovine and llama adenoviruses digested by enzyme *Hind* III.

S#	Sample Type	.....Kbp.....									
		B #	B #1	B #2	B #3	B #4	B #5	B #6	B #7	B #8	B #9	Total
01	OAV-1	9	8.80	6.21	4.84	3.63	2.44	1.75	1.60	1.31	0.88	31.46
02	OAV-2	8	10.0	5.7	4.51	3.78	3.31	2.53	1.42	1.24		32.49
03	OAV-3	9	8.08	5.88	4.54	3.71	2.85	2.18	1.59	1.21	1.00	31.04
04	OAV-4	9	6.62	5.35	4.74	4.23	3.38	2.63	2.10	1.69	1.25	31.99
05	OAV-5	5	15.2	9.41	4.01	2.71	2.23					33.56
06	RTS-42	7	11.1	5.58	4.45	4.11	2.85	2.32	1.81			32.22
07	OAV-6	UC										-
08	RTS-151	3	24.9	5.17	3.36							33.43
09	475-N	UC										-
10	32-CN	UC										-
11	47-F	8	12.1	4.73	4.04	3.34	2.70	2.22	1.7	1.62		32.45
12	LA-7649	5	16.3	5.40	4.10	3.27	1.95					31.02
13	LA-5330	4	14.2	8.87	4.65	3.50						31.22
14	OFC	-										
15	LMK-1	-										
16	MM-9780	6	23.1	9.41	6.55	4.36	2.32	2.02				
17	MM-0672	5	1.35	1.07	0.87	0.60	0.31					

Legends.

OAV	Ovine adenovirus	LA	Llama adenovirus
M Wt	Molecular Weight	Kbp	Kilobase pair
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LMK-1	Llama kidney cells	MM	Molecular Markers
-	No bands or not estimated	B #	Band Numbers

Table 6. The genome size of the ovine and llama adenoviruses serotypes, estimated on the basis of the migration of the RE fragments generated by four different restriction enzymes.

S#	Sample Type	.....Kbp.....				
		Bam HI	*Hind* III	*Pst* I	*Eco* RI	Mean
01	OAV-1	34.31	31.46	31.73	32.00	32.40
02	OAV-2	32.28	32.49	32.15	31.64	32.14
03	OAV-3	33.30	31.04	32.68	30.64	31.92
04	OAV-4	32.79	31.99	31.35	31.32	31.86
05	OAV-5	35.25	33.65	31.98	32.60	33.37
06	RTS-42	33.65	32.22	31.85	UC	32.57
07	OAV-6	33.20	UC	30.88	UC	31.94
08	RTS-151	32.72	33.43	31.60	UC	32.58
09	475-N	31.89	UC	31.71	UC	31.80
10	32-CN	32.62	UC	32.57	UC	32.60
11	47-F	UC	32.45	32.24	31.90	32.20
12	LA-7649	31.57	31.02	31.32	31.32	31.31
13	LA-5330	31.71	31.22	31.98	31.79	31.68
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 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 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. *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 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 5. *Eco* RI 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

DISCUSSION

Digestion of ovine and llama adenoviruses species with the four restriction enzymes resulted in different RE bands patterns that was attributed to their unique genome organization. These differences were expressed as variations in number and sizes of RE bands produced by each of the enzyme. Also, some strains showed difference in specificity to an individual enzyme i.e., they did not produce any bands and remain uncleaved. This observation suggests that variations in genome organization could be used to characterize a new species of the virus within the group.

The most successful RE that cleaved all the ovine adenovirus strains and produced 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, (Yousaf, 1993) to RTS-42, produced a 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 number and sizes of bands when compared to OAV-6. This supports the concept that serologically similar (Yousaf, 1993) strains could generally produces 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 (Pommer, 1991).

The second most successful restriction enzyme was *Bam* HI that digested all the strains except isolate 47F. This RE, although it did not produced many fragments for all viruses, yielded bands that were very visual and distinct. Isolate 475N and 32CN (serologically identical but distantly related to OAV-6 and RTS-151) produced RE patterns that 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 un-cut while both of OAV-5 and RTS-42 produced distinct RE patterns. This has been confirmed in another study (Pommer, 1991) 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 a distinctive RE fragments between themselves except for the isolate 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 the least successful in demonstrating the variations between all species of OAV. Most 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 LA7649 and LA5330, 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 cleaved both strains of llama adenoviruses and it did not cleave all the ovine adenovirus species.

Results of this study can be explained by the fact that, by serologically defining a difference between species of a same virus group, there is a relatively broad range of antibody titre that does 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 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 of RE pattern on the basis of digestion of a particular species of virus with certain number of restriction enzymes? More research in this field including testing other restriction enzymes which can elaborates 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, is 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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