CHARACTERISATION OF GASTRO-ENTERITIS-ASSOCIATED ADENOVIRUSES IN SOUTH AFRICA

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Objective. To analyse adenovirus (Ad) numbers and types associated with paediatric gastro-enteritis in South Africa.

Setting. Gauteng, 1994 - 1996.

Methods. A total of 234 paediatric diarrhoeal stool samples were screened for Ad using commercial enzyme-linked immunosorbent assays (ELISAs). Adenoviral isolates were typed, where possible, using restriction enzyme analysis.

Results. Ad was detected in 23 (9.8%) specimens, of which 8 (34.8%) were found by subgroup F-specific ELISA to contain Ad40 or 41. Six of these isolates were typed and 2 could not be typed. Of the remaining 15 specimens, 2 isolates had restriction profiles that did not correspond with known Ads, while 2 were identified as Ad31 and 1 as a subgroup C Ad. The remaining 10 specimens negative for Ad40/41 were non-cultivable and could not be typed.

Conclusions. The high percentage of non-cultivable Ads other than Ad40/41 is unusual, and may possibly indicate the prevalence of hexon variants of Ad40/41 or of emerging Ad types in South Africa.

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Diarrhoeal diseases are a major cause of death in infants and young children in South Africa. In 1986 gastro-enteritis was estimated to account for 27.5% of the total number of infant deaths. The most common aetiological agents of viral gastro-enteritis are the rotaviruses and adenoviruses.[1,2]

The adenovirus (Ad) serotypes most frequently associated with gastro-enteritis include those of subgenus A (types 12, 18 and 31) and subgenus F (types 40 and 41).[3-5] Subgenera A and F

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serotypes are therefore referred to as 'enteric' Ads based on their shared tropism for the gastro-intestinal tract.[6]

In cell culture, Ad40 and Ad41 grow poorly (relative to other serotypes) and are therefore referred to as 'fastidious' enteric Ads (FEAds).[1] The defectiveness of FEAd growth *in vitro* makes diagnosis of these viruses difficult. Types 40 and 41 are often diagnosed by presumptive methods based on the presence of large numbers of Ad particles in stool, as detected by electron microscopy, and the inability to culture these viruses in cell lines that support the growth of conventional Ads.[7]

Ad40 and Ad41 have been propagated in Chang conjunctival cells, Graham 293 cells, A549 cells and PLC/PRF/5 cells. However, differences in susceptibility to virus growth in different batches of the same cell line and variations in the growth of different virus strains make diagnosis using cell culture difficult.[5] The development of commercial ELISAs using monoclonal antibodies directed against subgroup-specific epitopes has facilitated diagnosis of Ad40/41 elsewhere,[6] but their specificity for South African FEAd strains is unknown.

Ad31 has been increasingly isolated during the past decade, and is significantly associated with paediatric gastro-enteritis.[4,6] The clinical features of Ad31 gastro-enteritis are indistinguishable from those of Ad40/41 gastro-enteritis.[8] The importance of Ad31 in paediatric gastro-enteritis in South Africa is not known.

This study examined the numbers and types of Ads detected in paediatric diarrhoeal specimens in South Africa.

MATERIALS AND METHODS

Cell lines

Chang conjunctival cells and the PLC/PRF/5 cell line were kindly supplied by C T Tiemessen (National Institute for Virology). Cells were cultured in minimal essential medium (MEM) with Hepes salts and 5 - 10% fetal calf serum, at 37°C. Minocyclin (2 µg/ml), penicillin (0.1 mg/ml) and streptomycin (0.1 mg/ml) were incorporated in the culture medium.

Viruses

Paediatric diarrhoeal stool specimens were obtained from the Medical University of Southern Africa, Ga-Rankuwa. Specimens were screened for Ads using a commercially available enzyme-linked immunosorbent assay (ELISA) (Biotrin International Adenovirus Antigen EIA). Samples with absorbances (450 nm) greater than 0.3 were regarded as positive (the high value was used to reduce the possibility of false positives). Ad-positive specimens were further screened for the presence of subgroup F Ads by ELISA (Cambridge Biotech Adenoclone — Type 40/41 EIA).

Infection of cells

Specimens were prepared as 10% weight/volume (w/v) suspension in MEM without serum, vortexed for 1 minute, then clarified by centrifugation at 12 000 *g* for 5 minutes. Clarified stool suspensions were inoculated onto duplicate subconfluent cultures of Chang conjunctival cells and PLC/PRF/5 cells, and incubated overnight at 37°C. Infected cells were washed with phosphate buffered saline, then maintained in MEM with 10% fetal calf serum.

Cells were harvested when maximum cytopathic effect (CPE) was observed (or 3 weeks post-infection when no CPE was observed), and subjected to three freeze-thaw cycles to release virus. Virus growth was monitored by ELISA (Biotrin International).

Extraction of virus from stool specimens

Virus was extracted directly from stool specimens by a modification of the procedure described by Buitenwerf *et al.*[9] Approximately 500 µl faeces were suspended in 1 500 µl of STE buffer (Tris-HCl 50 mM, NaCl 100 mM, EDTA 5 mM, pH 7.4), and vortexed for 1 minute with glass beads (75 - 150 µm). The suspension was clarified by centrifugation at 4 000 *g* for 10 minutes at 4°C. The supernatant was extracted with an equal volume of 1,1,2-trichloro-triflouro-ethane (Sigma) and centrifuged at 4 000 *g* for 10 minutes at 20°C. The aqueous layer was removed, and the virus precipitated by the addition of polyethylene glycol 6 000 to a final concentration of 10%, and 0.5M NaCl. After precipitation for 2 hours at 4°C, the virus was pelleted by centrifugation at 20 000 *g* for 20 minutes at 4°C, and was resuspended in 500 µl STE buffer.

Extraction of virus from harvested cell supernatant

Virus was extracted from infected cells after freeze-thawing and clarification at 4 000 *g* for 10 minutes at 4°C. Five millilitres of clarified supernatant was extracted with 1,1,2-trichloro-triflouro-ethane (Sigma) and the virus was precipitated using the above method.

Extraction of viral DNA

The pelleted virus was resuspended in 500 µl STE buffer. Proteinase K and SDS were added to a final concentration of 250 µg/ml and 1% respectively, and the reaction incubated for 30 minutes at 37°C. Protein was extracted once with TE-saturated phenol and once with phenol-chloroform (25:24:1 phenol: chloroform: isoamyl alcohol). Sodium acetate was added to a final concentration of 0.5M. Two volumes of absolute ethanol were added, and the DNA precipitated overnight at –70°C. The DNA was pelleted by centrifugation at 20 000 *g* for 30 minutes at 0°C, then resuspended in 20 µl TE buffer (pH 7.4).

DNA restriction analysis

Restriction analysis was performed with Sma I, Bam HI and Bgl II (Boehringer) at a concentration of 1 U/µl. Restriction proceeded at 37°C for 30 - 60 minutes in the recommended buffers supplied with the enzymes. Agarose gel electrophoresis

was performed in 0.8 - 1% agarose gels containing 0.5 µg/ml ethidium bromide, using TBE buffer (45 mM Tris-Borate, 1 mM EDTA), pH 8.0.

Transmission electron microscopy

Stool specimens were prepared as 10% (w/v) suspensions in distilled water, and centrifuged at 12 000 *g* for 10 minutes. The clarified suspension was ultracentrifuged at 53 000 *g* for 90 minutes. Tissue culture adapted virus was concentrated by addition of PEG 6 000 as described previously. The virus was resuspended in distilled water, and stained with 3% w/v phosphotungstic acid, pH 6.8. Samples were viewed under a Joel JEM-100S transmission electron microscope.

RESULTS

A total of 234 paediatric diarrhoeal stool samples were screened by the group-specific ELISA (Biotrin), of which 23 (9.8%) were found to be positive for Ad (Table I). These 23 samples were further screened using the subgroup F-specific ELISA (Adenoclone).

Of the 23 samples, only 8 (34.8%) were found to be positive for Ad40 and Ad41. Where sufficient sample was available, DNA was extracted directly from stool specimens. Where direct extraction was impossible, samples were used to infect Chang conjunctival and/or PLC/PRF/5 cells, and viral growth was monitored by examination for CPE and/or group-specific ELISA of tissue culture supernatant.

Of the 8 specimens positive for Ad40/41, 3 isolates were identified as Ad40, and 3 as Ad41 based on Sma I restriction profiles. The remaining 2 isolates could not be typed.

Attempts to culture Ad from the 15 specimens negative for Ad40 and Ad41 failed in all but 4 cases. Restriction analysis using Sma I was performed on each of these 4 isolates and on a single non-cultivable isolate extracted directly from the stool sample.

Two cultivable isolates were typed as Ad31 by restriction analysis using Sma I, Bam HI and Bgl II, and a single cultivable isolate was identified by restriction analysis as belonging to subgroup C, but was not typed.

Two isolates showed DNA banding profiles that did not correspond to that of any known Ad type (Figs 1 and 2).[10,11] Electron microscopy was used to confirm the presence of Ad in these isolates (Fig. 3). However, one of these isolates may comprise part of a dual infection as the total DNA length (40kB) exceeds that of an Ad (Fig. 1, lane 5 and Fig. 2b).

The remaining isolates were non-cultivable in Chang conjunctival and PLC/PRF/5 cell lines, and could therefore not be typed. Extraction directly from stool specimens was unsuccessful. These specimens were examined by electron microscopy and Ad particles were seen in only 4 of the 10 untyped specimens.

Table I. Analysis of Ad numbers and types detected in stool specimens

	Number of isolates	Percentage
Ad	23/234*	9.8% of total diarrhoea
Subgroup F Ads	8/23	34.8% of Ads
Type 40 (by RFLP)	3/23	13.0%
Type 41 (by RFLP)	3/23	13.0%
Untyped†	2/23	8.7%
Subgroup A Ads		
Type 31	2/23	8.7%
Subgroup C Ads		
Untyped	1/23	4.3%
Untyped (non-subgroup F)	12/23	52.2%
Unknown DNA profiles by restriction analysis	2/23	8.7%
No DNA profile†	10/23	43.5%

* Total number of diarrhoeal specimens screened by ELISA.
†Isolates were non-cultivable and virus extraction from stool was unsuccessful.
RFLP = restriction fragment length polymorphism.

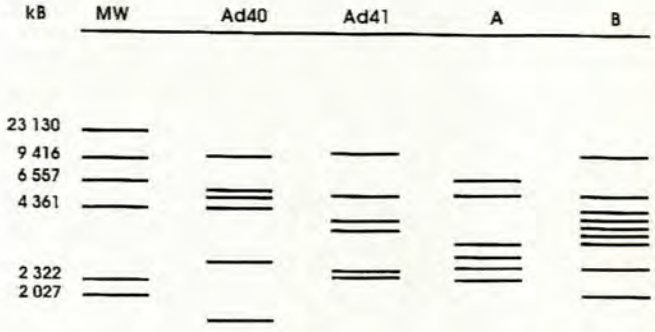

Fig. 1. Diagrammatic representation of 0.8% agarose gel electrophoresis of Sma I restricted viral DNA. Lane 1 — molecular weight marker (λDNA restricted with Hind III), lane 2 — Ad40, lane 3 — Ad41, lane 4 — untyped isolate A, lane 5 — untyped isolate B.

DISCUSSION

Ads have been associated with 3.1 - 13.5% of cases of paediatric diarrhoea in studies from Asia, Europe, and North and South America (Table II). The subgroup F Ads are reported to comprise between 34.8% and 100% of these Ads. In previous studies in South Africa, Ad40 and Ad41 were associated with 4.3 - 13.2% of paediatric gastro-enteritis cases,[12,13] and were found to be the predominant types of Ads associated with diarrhoea (65.6%).

The number of Ads and specifically subgroup F Ads detected in diarrhoeal stool specimens in this study is therefore consistent with previous reports. However, the observation that the subgroup F Ads in this study comprised only 34.8% of all Ads detected in stools differs from previous reports. The number of Ads that could not be typed (52.2%) also differs significantly from reported numbers, suggesting the possibility of a shift in the prevalence of specific Ad types in South Africa.

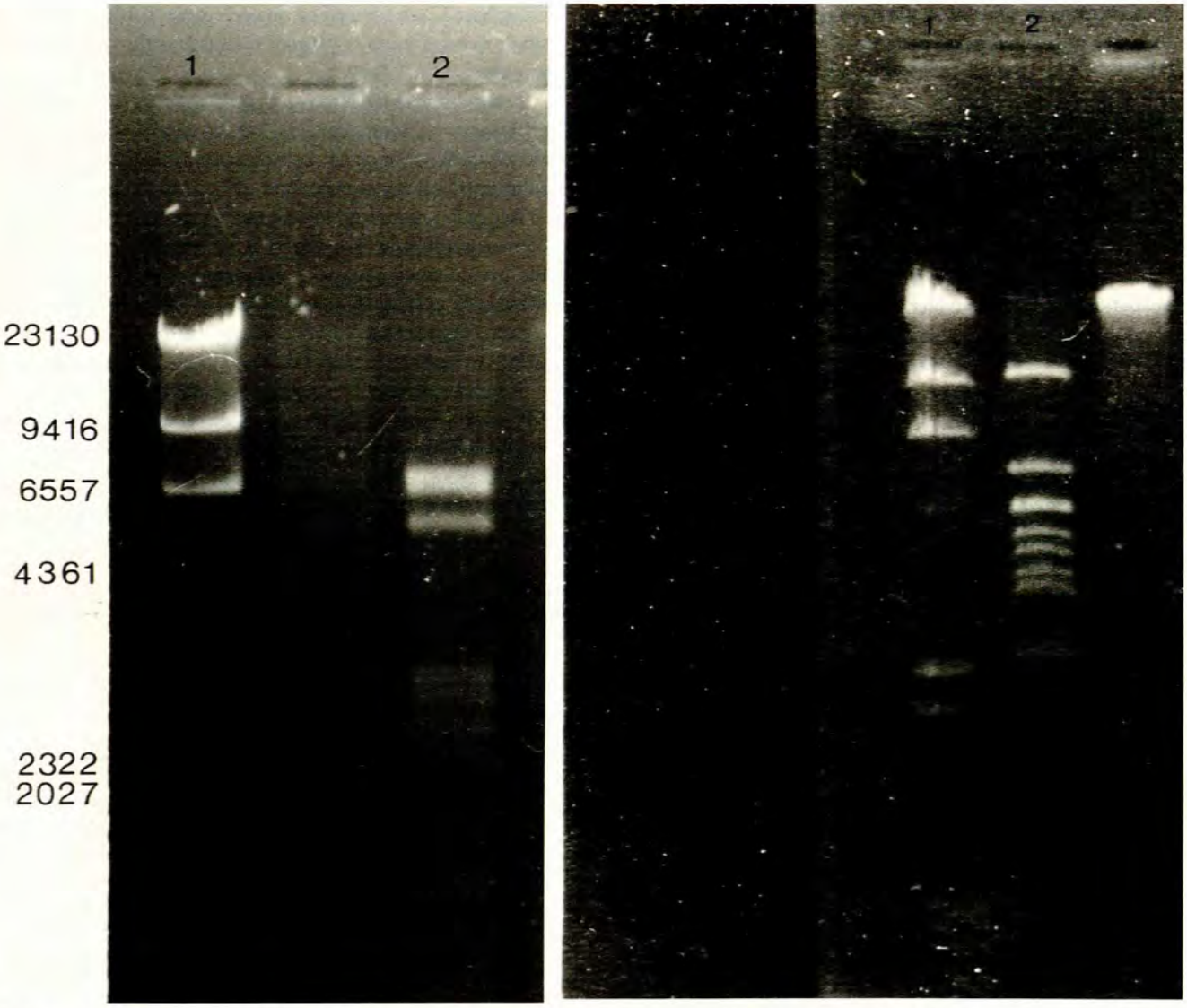

Fig. 2. Agarose gel electrophoresis (0.8%) of Sma I restricted viral DNA. Left: lane 1 — molecular weight marker (λDNA restricted with Hind III), lane 2 — untyped isolate A. Right: lane 1 — molecular weight marker (λDNA restricted with Hind III), lane 2 — untyped isolate B.

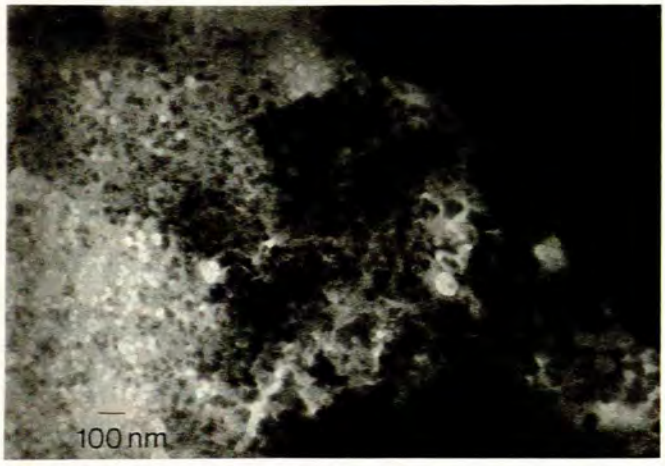

Fig. 3. Electron microscopy of PEG precipitated virus (isolate A) propagated in PLC/PRF/5 cells.

Two Ad isolates were identified by restriction analysis as Ad31. This is, to our knowledge, the first report of Ad31 in South Africa, although the association of Ad31 with gastro-enteritis is well established elsewhere.[4,6]

The isolation of a single subgroup C isolate is consistent with other studies. Although subgroup C Ads have not been aetiologically associated with gastro-enteritis, Ads 1, 2 and 5 are occasionally recovered from stools.[4,12]

The 10 non-cultivable specimens negative by Adenoclone ELISA for Ad40/41 are unusual. Although Ads were detected by electron microscopy in only 4 of the 10 isolates, it is possible that viruses were excreted at levels too low to be detected by electron microscopy in the remaining 6 specimens.

The Adenoclone immunoassay employs monoclonal antibodies (MAbs) directed against type-specific epitopes on Ad40 and Ad41 hexon, and therefore detects subgroup F Ads.[14]

Table II. Reported occurrence of gastro-enteritis-associated Ads

Country	% Ad of total diarrhoea	% Ad40/41 of total diarrhoea	Percentage of total Ads			
			Ad40/41	Ad31	Other types	Untyped
Australia (1981 - 1992)[20]	3.1	3.1	100	0	0	0
Sweden (1981)[2]	13.5	7.9	58.9		27*	14.3
Canada (1983 - 1986)[4]	ND	ND	54.9	17.8	25.2	2.1
Brazil (1988 - 1998)[21]	10	3.75	37.5	0	37.5	25
Canada (1988 - 1992)[16]	9.9	4.9	49	6.1	30.1	14.8
Guatemala (1987 - 1988)[22]	ND	14.0	ND	ND	ND	ND
South Africa (1985 - 1986)[13]	ND	13.2	ND	ND	ND	ND
South Africa (1982 - 1983)[12]	6.5	4.3	65.6	0	1.6	32.8
South Africa (1994 - 1996)†	9.8	3.4	34.8	8.7	4.2	52.2

* Includes 'conventional' Ad types 1 - 39.
† This study.
ND = not done.

The neutralisation epitopes of Ads are composed of serotype-specific residues in 7 hypervariable regions (HVRs) in the hexon region. These HVRs are subject to recombination and an unusually high rate of single base mutations.[15] The outer surface of the hexon is thought to be a neutralisable determinant and is most prone to antigenic drift. Thus highly specific MAbs to surface components of the hexon may be susceptible to loss of reactivity with changes in epitopes under immunological selection pressure.[16]

The emergence of hexon variants has previously been shown to compromise the value of existing immunoassays.[17-18] Scott-Taylor *et al.*[17] identified several Ad41 strains that escaped detection by an early version of the Adenoclone 40/41 kit (Cambridge Bioscience). The modified kit now detects the Ad41 strains circulating in North America,[17] but the emergence of new variants remains a potential problem.[6]

The fastidiousness of 10 untyped Ad isolates *in vitro* suggests that these could be subgroup F Ads. It is possible that variants of Ad40/41 prevalent in South Africa may have undergone variations in that region of the hexon to which the MAbs used in the Adenoclone kit are directed.[14] They may therefore not be reactive with those MAbs and so would not be detected by the Adenoclone 40/41 kit.

Another possibility is that these non-cultivable and non-Ad40/41 isolates are newly emerging Ad types. This is further suggested by the isolation of 2 isolates with DNA restriction profiles that do not correspond to any known Ads.

Genetic variability within the Ad group is considerable. The emergence of 7 new types (Ads 42 - 49), isolated almost exclusively from AIDS patients, has been reported since the mid-1980s. In addition to new serotypes and variants, intertypic strains called intermediates have been described in increasing numbers, particularly from AIDS patients.[19]

Homologous recombination between closely related strains or serotypes has consistently been proposed as the primary evolutionary mechanism for Ads.[19] The genetic variability of the Ads is further affected by the presence of HVRs in the hexon region, and the possibility of illegitimate recombination events (involving DNA of limited homology, with short direct repeats at junctions) in such regions.[19]

No epidemiological studies of Ads in South Africa have been performed since 1989.[13] It is possible, given the extreme variability of Ads, that previously unidentified fastidious Ads have become increasingly prevalent in South Africa during the past decade.

These results indicate that the use of either the Biotrin or the Adenoclone ELISAs as diagnostic tools in South Africa may be inappropriate. There appear to be significant differences between the enteric Ads prevalent in South Africa and those commonly reported worldwide. This indicates a need for further characterisation of Ads associated with gastro-enteritis in South Africa.

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