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Recognition of adenovirus types in faecal samples by Southern hybridization in South Australia

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SUMMARY

The distribution of adenovirus types in faecal samples of patients with suspected viral gastroenteritis from South Australia was determined during the 12-month period, July 1991–June 1992. There were 3299 samples tested and 226 (6·9%) were positive for adenovirus by enzyme immunoassay. Of these 226 samples, 154 (68%) were typed directly using virus DNA extracted from the faecal samples according to the *Sma* I, *Hin*d III and *Bst*E II restriction patterns and Southern hybridization analysis with pooled viral genomic DNA probes. In this group, 86% of the samples were from patients who were < 3 years of age. Enteric adenovirus types 40 and 41 accounted for 20 and 40% respectively, of these samples, and types 1, 2, 3, 5, 6, 7 and 31 comprised the remainder. Type 40 was detected mainly in the winter and spring periods, and type 41 predominated in the autumn period. The majority of the non-enteric types were found during the late winter and spring periods.

INTRODUCTION

Adenoviruses, in particular types 40 and 41, have been clearly established as important aetiological agents of viral gastroenteritis and are second only in frequency to rotavirus [1–3]. However, it has been known for many years that other adenovirus types may be detected in the faeces of patients without gastroenteritis or in asymptomatic individuals [1]. After the introduction of an adenovirus enzyme immunoassay as a routine test in our laboratory, it was noted that the incidence of adenovirus in faecal specimens was relatively constant throughout the year, compared to the rotavirus incidence which showed a peak during the winter period (unpublished observations). Preliminary tests with culture of faecal specimens in HEp-2 and 293 cell lines [4] showed that some of these specimens were positive for the fastidious or enteric adenovirus types 40/41 and other adenovirus types. In view of these results, and reports from other laboratories which showed a high incidence of the enteric adenoviruses [1, 2], we wanted to determine the incidence and distribution of adenovirus subtypes in the

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faecal samples of patients with suspected viral gastroenteritis in South Australia. Faecal samples were submitted to our laboratory for routine tests by enzyme immunoassay (EIA) for rotavirus and adenovirus. Samples which were positive for adenovirus by EIA were typed according to the *Sma* I, *Hin*d III and *Bst*E II restriction patterns [5] following Southern hybridization with pooled genomic viral DNA probes from the six adenovirus groups. This study showed that adenovirus types 40 and 41 accounted for 60% (20 and 40% respectively) of the samples which were positive for this virus in the EIA and with detectable viral DNA by Southern hybridization.

MATERIALS AND METHODS

Clinical specimens

The faecal samples tested in this study were submitted to the laboratory for routine viral examination from patients with gastroenteritis. There were 3299 samples collected during the period July 1991–June 1992. The majority (approximately 90%) were from patients under 5 years of age. Suspensions of faeces (10% w/v) were made in TE buffer (10 mM TRIS/1 mM EDTA) and clarified by centrifugation at 150 g for 5 min at room temperature (RT).

Enzyme immunoassays for rotavirus and adenovirus

The clarified suspensions were tested for rotavirus by enzyme immunoassay (EIA) using antisera obtained from Dakopatts (Denmark) as previously described [6]. The adenovirus EIA used was similar to the indirect format described previously [7] using rabbit and guinea-pig antibodies prepared with purified adenovirus [8]. Rabbit antiserum was used as the capture antibody, with the guinea-pig antiserum as the detecting antibody. The samples were inoculated into a pair of antiserum coated wells and an adjacent pair of non-immune rabbit serum coated wells (control). The inoculated wells were incubated O/N at RT. Unbound sample was removed by five washes in PBS/T (PBS with 0·05% Tween 20). Guinea-pig anti-adenovirus serum was then added and incubated for 1 h at 37 °C, followed by five washes to remove unbound guinea-pig detector antibodies. The bound detector antibodies were detected with rabbit anti-guinea-pig immunoglobulins conjugated with horseradish peroxidase (Dakopatts, Denmark) for 1 h at 37 °C. The chromogen used for the enzymic reaction was 3,3′,5,5′- tetramethyl benzidine dihydrochloride (TMB) according to the manufacturer's instructions (Sigma). Each reaction volume used throughout the EIA was 100 μl. The optimal dilutions of the antisera used were determined by previous titrations. The polyclonal antibodies used in the EIA were shown to react with adenovirus types from the six different groups (data not shown). The cut-off criteria for the adenovirus EIA were calculated from the ratio of the average absorbance readings of the specific antibody-coated wells to the average absorbance readings of the non-immune serum coated wells and from the difference between the absorbance of the specific antibody-coated wells and non-immune serum coated wells. A sample was considered positive for adenovirus when the ratio was greater than 2·0 and the difference was greater than 0·1.

Preparation of prototype adenovirus DNA

Prototype adenoviruses representing each of the six groups were obtained from the American Type Culture Collection. Ad18 and 31 (group A), Ad3, 11, 16 (group B), Ad2 and 5 (group C) Ad9 and 13 (group D), Ad4 (group E) were cultured in A549 cells, while Ad40 (Dugan) and 41 (Tak) (group F) were cultured in 293 cells [4]. Cultures were maintained until cytopathic effects developed in 50–75% of the cell monolayer. The cells were dislodged and harvested by knocking the flasks. The adenovirus virions were purified from the cells as previously described [8]. Adenovirus DNA was then extracted using the Hirt procedure [9, 10].

Preparation of adenovirus DNA from faecal samples

DNA was extracted from a 1 ml aliquot of the clarified 10% faecal suspensions using the Hirt procedure [9, 10] and resuspended in 40 μl of distilled water. The DNA was stored at −20 °C until it was analysed by restriction endonuclease digestion.

Restriction endonuclease digestion and gel electrophoresis

Ten microlitre volumes of the extracted DNA were digested with 20 units of restriction endonuclease, either *Sma* I and *Hin*d III (Amersham International) or *Bst*E II (Boehringer Mannheim) for 2 h at 37 °C. The digested products were applied to a 1% agarose gel containing 40 μg/ml ethidium bromide and electrophoresed at 2·5 V/cm (4 h at 75 V) in TAE running buffer (0·04 M TRIS-acetate, 0·001 M EDTA).

Southern blot hybridization

Following agarose gel electrophoresis, the DNA was transferred to a nylon membrane (Hybond N+, Amersham International) by Southern blotting [11]. The DNA was immobilized on the membrane by ultraviolet irradiation for 2 min on an Ultra-Lum Transilluminator (Ultra-Lum Inc., Carson, California). The membrane was pre-hybridized for 3–4 h at 42 °C in 10 ml of hybridization buffer [12]. The buffer was removed and replaced with 6 ml of hybridization buffer containing heat-denatured digoxigenin labelled probes from adenovirus types representing each of the six adenovirus groups (Ad3, 4, 5, 9, 31, 40, 41). The membranes were hybridized overnight at 42 °C. The buffer was then removed and stored at −20 °C. This buffer was re-used for a further four hybridization reactions. The membrane was then washed as described in [11] except that the stringent wash used 0·1 × SSC and 0·1% SDS. The membrane was then blocked with 0·5% skimmed milk in Buffer I (0·15 M-NaCl, 0·1 M TRIS) for 1·5 h at RT. The hybridized probe was detected by horseradish peroxidase-conjugated sheep anti-digoxigenin antibody (1/2500) (Boehringer Mannheim) in 0·25% blocking agent (Amersham International) diluted in Buffer I for 30 min at RT. Following two washes of the membrane in Buffer I for 15 min, the membrane was reacted with the Enhanced Chemiluminescent (ECL) detection system according to the manufacturer's instructions (Amersham International). The membrane was then exposed to X-ray film (X-Omat K, Eastman Kodak Company, NY) at RT for 1–20 min before developing.

Non-isotopic labelling of prototype adenovirus DNA

Purified adenovirus DNA (500 ng) was labelled with digoxigenin-dUTP using a random primer DIG DNA Labelling Kit (Boehringer Mannheim) according to the manufacturer's instructions. The viral DNA was labelled for 18 h at 37 °C.

Optimization and sensitivity of prototype adenovirus probes

Adenovirus DNA from members of the same group is highly homologous but there is only limited homology between members of the different groups [13]. Consequently it was necessary to establish the optimum concentration of labelled probes from the various groups to be included in the hybridization buffer for Southern blot analysis.

Each prototype adenovirus probe was used in hybridization assays to determine the degree of homology with the homologous DNA, DNA from other members of the same group and DNA from members of the other groups. Each labelled probe was found to give 100% homology with both the homologous DNA and DNA from members of the same group, but only limited homology with DNA from members of the other groups (data not shown). Thus it was necessary to include labelled DNA from each of the six groups in the hybridization buffer to ensure that all the types could be identified.

Titrations with homologous DNA and DNA from members of the same group showed that the detection limit of each probe was in the range 1–10 pg adenovirus DNA.

RESULTS

Incidence of non-enteric adenovirus types

Fig. 1 shows the monthly distribution of adenovirus positive samples as detected by EIA and of adenovirus types by Southern hybridization during the study period. There were 3299 samples tested in the EIA and 226 (6·9%) were positive for adenovirus. Of these 226 samples, 7 were also positive for rotavirus by EIA (see below). The peak incidence of adenovirus occurred during the late summer/early autumn (February–April) period. Of the 226 samples positive for adenovirus by EIA, 154 (68%) were typed by Southern hybridization. The non-enteric adenovirus types were found throughout the year, with Ad1 the predominant type detected. The peak incidence of this type occurred during October 1991 (spring season). Ad31, which has also been implicated in viral gastroenteritis [3], accounted for only 2% (3/154) of the samples typed by Southern hybridization. The frequencies of the adenovirus types detected are shown in Table 1.

Incidence of enteric adenovirus types

The enteric adenovirus types 40 and 41 accounted for 60% (31/154, 20%; 61/154, 40% respectively) of the adenoviruses typed in this study (Table 1). Ad40 was found predominantly throughout the winter and spring periods (July–November 1991 and in June 1992) (Fig. 1). The incidences of Ad40 detected in the summer months (December 1991–February 1992) were lower than the winter and spring periods. There was no Ad40 detected in March and May 1992.

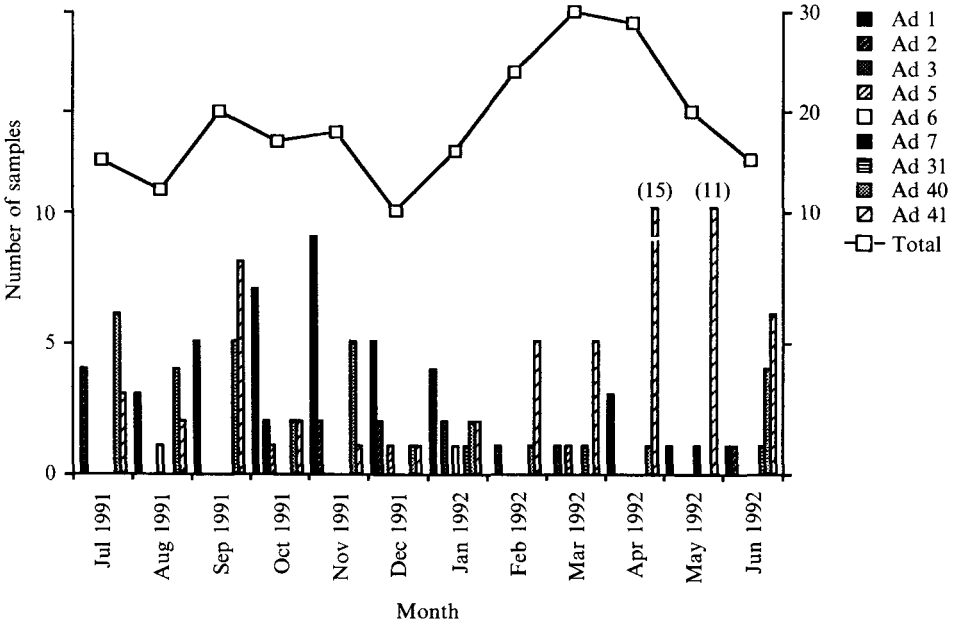

Fig. 1. Detection of adenoviruses by EIA and distribution of adenovirus types by Southern hybridization during the period July 1991–June 1992.

Table 1. *Adenovirus types in faecal samples identified by Southern hybridization during July 1991 to June 1992*

Adenovirus type	Number (%)
41	61 (40)
40	31 (20)
31	3 (2)
1	38 (25)
2	11 (7)
3	4 (2·5)
5	3 (2)
6	2 (1)
7	1 (0·6)
Total	154 (100)

Ad41 was found throughout the year, unlike Ad40 (see Fig. 1). During the summer months, the incidence of Ad41 was lower (8/61, 13%) compared to that observed during the other periods of the year. The highest incidence occurred during April–June 1992 (32/61, 52%).

Adenovirus types in faecal samples

Adenovirus types 3, 5, 6, 7, 31, 40, 41 (Figs. 2, 3) were identified by the *Hind* III and *Sma* I cleavage patterns. However the *Hind* III and *Sma* I cleavage patterns could not clearly differentiate between types 1 and 2 as these latter types showed similar restriction patterns with these enzymes. The *Sma* I digestion patterns for the prototype Ad1 and Ad2 are identical and the *Hind* III patterns

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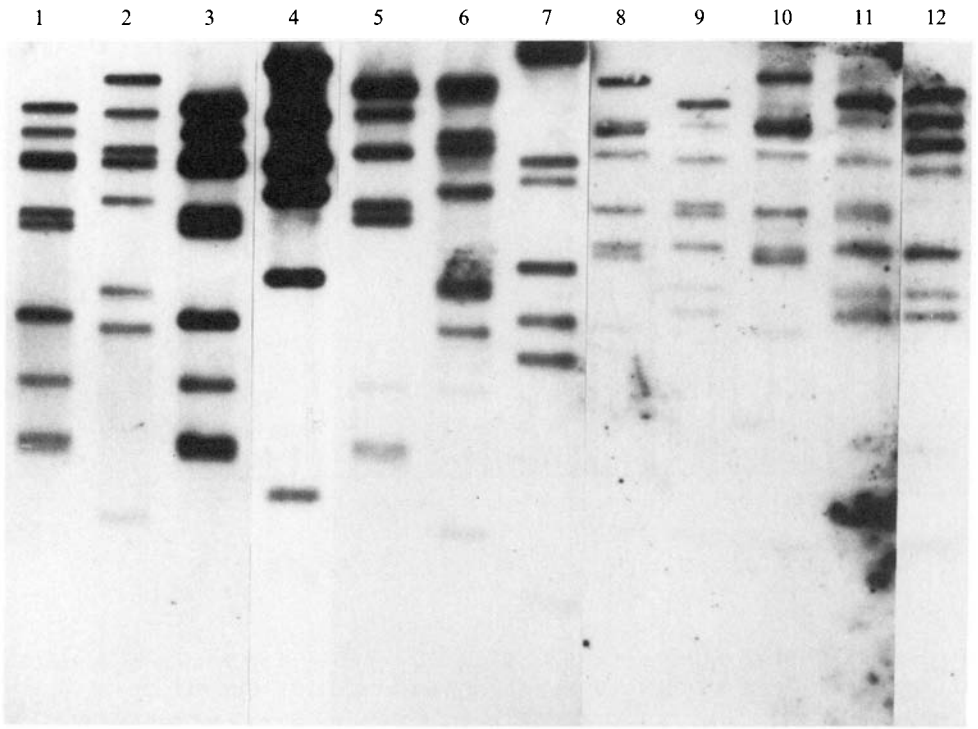

Fig. 2. Restriction enzyme digestion patterns of adenovirus types 3, 5, 6 and 7 from faecal samples as detected by Southern hybridization. Lanes 1, 3, 5, 8, 10 *Hin*d III, lanes 2, 4, 6, 9, 11, 12 *Sma* I, lane 7 *Bst*E II. Lanes 1–2 prototype Ad3, lanes 3–4 Ad3 from a clinical sample, lanes 5–7 Ad7 from a clinical sample, lanes 8–9 prototype Ad5, lanes 10–11 Ad5 from a clinical sample, lane 12 Ad6 from a clinical sample.

are very similar [5]. In order to distinguish between Ad1 and Ad2, the samples were digested with *Bst*E II (Fig. 4*a*, *b*).

Unlike other reports [3, 14–16], the cleavage patterns of the Ad41 type in our study population did not vary throughout the period, but were different from the prototype Tak for both *Hin*d III and *Sma* I digestion (Fig. 3, lanes 11, 13 and lanes 12, 14 respectively) [5, 15]. A comparison of the cleavage patterns of the South Australian type 41 with the variants reported by van der Avoort and colleagues [15] indicated that the type is consistent with their most frequent variant, D12.

Two variants of Ad40 were detected, although the majority of samples (30/31) yielded the cleavage patterns shown in Fig. 3, lanes 7–8, while the remaining one sample showed unique cleavage patterns for both enzymes (Fig. 3, lanes 9–10). The variants resembled the prototype Dugan strain (Fig. 3), [5, 15].

Consistent with previous reports [3, 5], the group C adenoviruses, Ad1 and Ad2, showed the most variation in digestion patterns. Each type showed 3 variant patterns (Fig. 4*a*, Ad1; Fig. 4*b*, Ad2; lanes 4–6, 7–9, 10–12) compared to the prototype strains [5]. Variations were seen in the *Hin*d III or *Sma* I restriction patterns but not in the *Bst*E II patterns. The majority of Ad1 variants yielded the patterns shown in Fig. 4*a*, lanes 4–6, while the majority of Ad2 variants yielded the patterns shown in Fig. 4*b*, lanes 10–12.

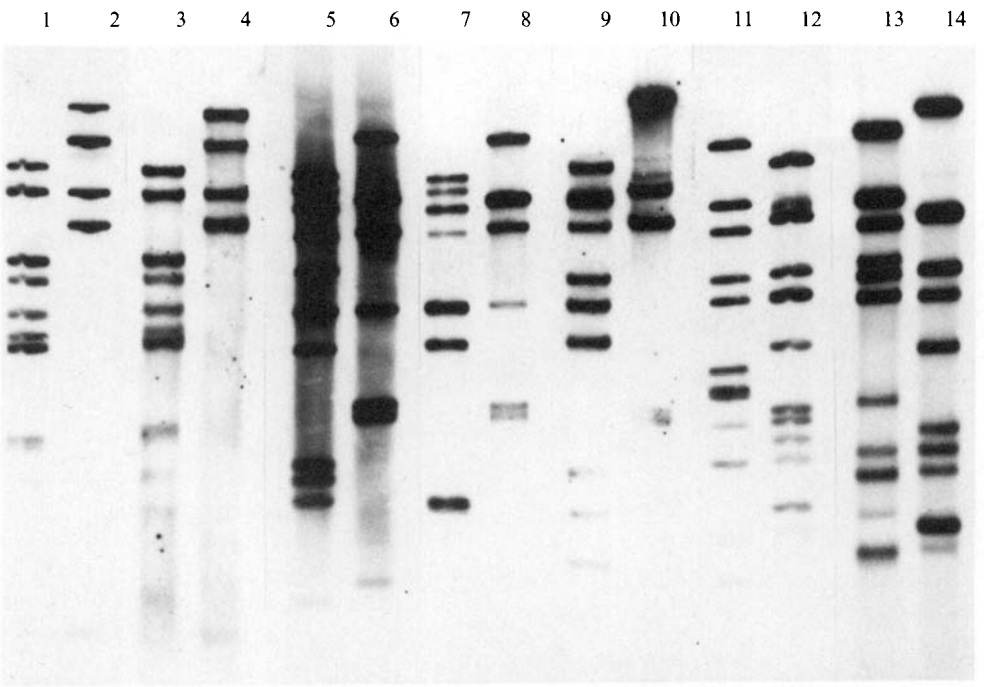

Fig. 3. Restriction enzyme digestion patterns of adenovirus types 31, 40 and 41 in faecal samples detected by Southern hybridization. Odd numbered lanes *Hind* III, even numbered lanes *Sma* I. Lanes 1–2 prototype Ad31, lanes 3–4 Ad31 from a clinical sample, lanes 5–6 prototype Ad40 (Dugan), lanes 7–8, 9–10 two Ad40 variants from clinical samples, lanes 11–12 prototype Ad41 (Iak), lanes 13–14 Ad41 from a clinical sample.

DISCUSSION

By extracting adenovirus DNA directly from faecal samples and using Southern hybridization with pooled genomic probes, adenovirus types were identified in 68% (154/226) of samples positive for this virus by an in-house EIA. This figure is comparable to those previously reported for direct DNA extraction methods using polyacrylamide gel electrophoresis and silver staining (14, 17). Although the typing of adenovirus by restriction enzyme digestion and ethidium bromide stained agarose gels have been used successfully (10, 18), we used Southern blot analysis in order to maximize sensitivity and to circumvent the difficulty with the interpretation of some ethidium bromide stained agarose gels, in particular with samples that have high contaminating levels of cellular DNA. Of the 154 samples typed, 60% were typed as the fastidious or enteric adenovirus types 40 and 41. Ad41 (40%) accounted for twice the number of cases as Ad40 (20%). Other studies [3, 14, 15, 19, 20–22] have shown that Ad41 is the predominant type associated with gastroenteritis and have also shown a decrease in the incidence of Ad40 as a causative agent. However, Brown [3] reported an increase in Ad40 during 1987/88 and Bates [22] also reported an increase in Ad40 cases from 1986/7–1988/9. The relatively high incidence of Ad40 in South Australia suggests that this type may be a significant contributor to adenovirus gastroenteritis.

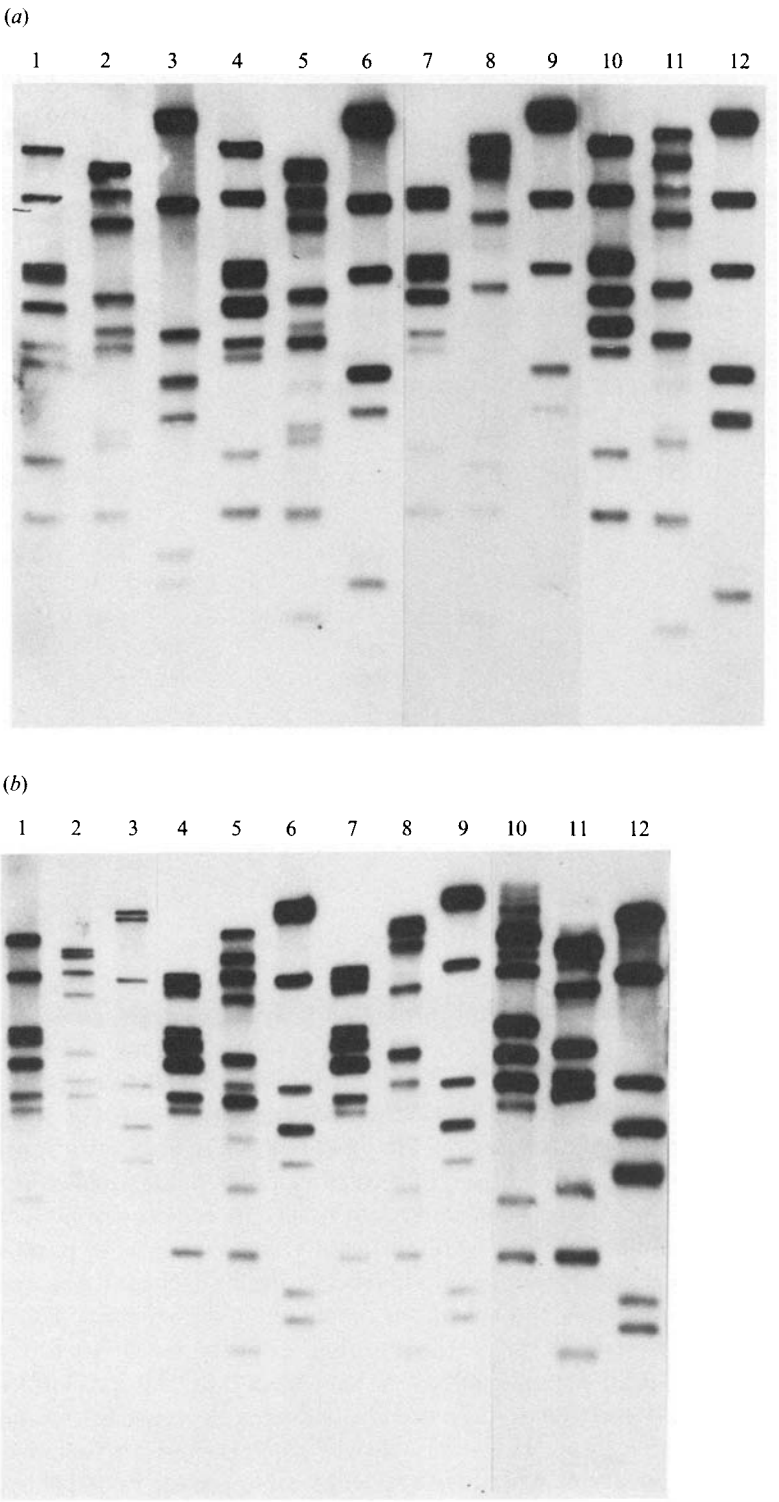

Fig. 4*a*, *b*. For legend see opposite.

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In the subpopulation of 226 samples which were adenovirus EIA positive, there were 72 samples (32%) which did not yield detectable Southern hybridization signals. On repeat extractions of viral DNA from these samples no hybridization signals were obtained. It is not known what factors could have caused these discordant findings between the EIA and Southern hybridization results. It is unlikely that the pooled probes used did not react with a particular type as preliminary results showed that the probes reacted with adenoviral DNA from all six groups. During the validation of the EIA, adenoviruses from the six groups were shown to be reactive in the assay. It may be possible that the EIA was detecting viral antigens shed from non-enteric sites (such as the respiratory tract). In addition, the levels of viral DNA may have decreased (below the sensitivity limit of the Southern hybridization) more rapidly than antigen levels during the period of storage. There was no known detrimental storage conditions which could have contributed to adverse effects on the stability of the viral DNA during the study period. The sensitivity limit of our Southern hybridization assay (*c.* 10 pg of viral DNA) was similar to that reported by Hyypia [23]. In this group of 72 samples, the EIA specific absorbance values (absorbance values of antibody-coated wells minus non-immune serum-coated wells) were in the range 0·12–3 (average 0·84, S.D. 0·64). There was no particular pattern of EIA absorbance values with these specimens.

Unlike previous studies [3, 14–16], no variants were detected in the 61 samples which were positive for Ad41. However, as this study was conducted during a 12-month period only, compared to the longer time periods of the previous studies, it is possible that the emergence of variants may not have been detected. Two variants of Ad40 were detected, although the majority (30/31) showed the same *Hind* III and *Sma* I cleavage patterns. The remaining one sample had different cleavage patterns for both restriction enzymes. Both variants showed similarities to the Ad40 prototype Dugan [5].

The presence of the enteric adenovirus types throughout the year, although at slightly higher levels during the winter/early spring and autumn months (see Fig. 1), is similar to the incidence recently reported in the Netherlands [20]. The higher incidence of Ad41 observed during the autumn season is similar to that reported by Shinozaki and colleagues [21]. However, their findings and that of ours differ from the study by Bates and colleagues [22] who showed that there was no particular seasonal pattern of infection by Ad40/41. It is likely that our findings reflect the shorter 12-month period of study rather than that observed by other laboratories which analysed incidences over a period of 5–6 years [21, 22].

In our study population *c.* 90% of the patients were under 5 years of age. It was thus not surprising that 86% (133/154) of the adenovirus positive samples (by Southern hybridization) were from patients who were under 3 years of age

Fig. 4. (*a*) Restriction enzyme digestion patterns of adenovirus type 1 variants in faecal samples detected by Southern hybridization. Lanes 1, 4, 7, 10 *Hind* III, lanes 2, 5, 8, 11 *Sma* I and lanes 3, 6, 9, 12 *Bst*E II. Lanes 1–3 prototype Ad2, lanes 4–6, 7–9, 10–12 three Ad1 variants from clinical samples respectively. (*b*) Restriction enzyme digestion patterns of adenovirus type 2 variants in faecal samples detected by Southern hybridization. Lanes 1, 4, 7, 10 *Hind* III, lanes 2, 5, 8, 11 *Sma* I and lanes 3, 6, 9, 12 *Bst*E II. Lanes 1–3 prototype Ad2, lanes 4–6, 7–9, 10–12 three Ad2 variants from clinical samples respectively.

(data not shown). This observation compares with that of Shinozaki and colleagues [21] who reported that 80% of adenovirus positive samples were from those under 2 years of age.

Rotavirus was detected in 7/226 adenovirus positive samples. In the group of seven rotavirus positive samples, Ad40 was detected in one sample, Ad41 was detected in a separate sample, Ad1 was detected in three other samples and two samples did not yield detectable adenovirus DNA. The presence of enteric adenovirus types 40 and 41 in two of the samples may suggest dual infections with adenovirus and rotavirus. Such dual infections have been reported previously [24]. The three samples with rotavirus and Ad1 may represent shedding of Ad1, possibly from the respiratory tract (see also [1]).

In summary, this study established the importance of enteric adenovirus types 40 and 41 associated viral gastroenteritis in South Australia. The use of non-isotopic Southern blot analysis of viral DNA extracted directly from faecal samples provided a useful technique for the analysis of adenovirus types in clinical samples.

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