
Infection by Enteric Adenoviruses, Rotaviruses, and Other Agents in a Rural African Environment

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From February 1985 to January 1986, 432 stool samples, 310 from rural African children with diarrhea and 122 from controls, were analysed for the presence of enteric viruses known to be associated with diarrhea. Group A rotavirus ELISA indicated 12.9% positivity among patients and 2.5% positivity among controls. Only 23 of the 43 rotavirus ELISA-positive stools were also positive by electron microscopy. Nine children, three of whom were controls, were found to be shedding coronavirus-like particles, detected by electron microscopy. Stools from all but one of the nine children had been taken within 1 month of each other. Dot-blot hybridization tests for the presence of Ad40 or Ad41 DNA revealed 44 positive stools, 41 of which were from patients (13.2% positivity). Only three of the Ad40- or Ad41-positive stools by DNA hybridization were positive by electron microscopy, and only these three strains could be grown in semipermissive Chang conjunctival cells and their identity checked by restriction enzyme analysis. Further attempts to rescue the other strains using a helper virus failed, but nine of the stools proved positive by ELISA using a subgroup F-specific monoclonal antibody. On the basis of the DNA hybridization results alone, subgroup F adenoviruses were encountered as frequently as rotavirus in the study and were significantly associated with diarrhea, although the viability and intactness of virus particles by the time of laboratory analysis appeared to be very low.

KEY WORDS: adenovirus 40, adenovirus 41, coronavirus, diarrhea, enteric infection, Africa

INTRODUCTION

Rotaviruses are widely accepted to be the most common viral cause of infantile gastroenteritis [Flewett et al., 1974; Kapikian and Chanock, 1985] and in developing countries, only enterotoxigenic *Escherichia coli* can rival rotavirus as an important etiologic agent of

diarrhea [Du Pont, 1984]. Unlike rotavirus, the Norwalk agent appears to infect mainly school-age children and adults and is often associated with water- or food-borne outbreaks of gastroenteritis [Kaplan et al., 1982]. The role of other viruses in causation of acute gastroenteritis is less clear. Some controlled studies have found coronavirus-like particles to be significantly associated with diarrhea [Caul et al., 1975; Vaucher et al., 1982; Gerna et al., 1985; Rettig and Altshuler, 1985], whereas other reports have revealed similar rates of shedding among asymptomatic individuals [McNaughton and Davies, 1981]. The role of subgroup F adenoviruses (Ad40 and Ad41) in the causation of diarrhea is better understood, with three controlled studies to date indicating a significant association with disease [Yolken et al., 1982; Uhnnoo et al., 1984; Brandt et al., 1985]. There have also been reports of outbreaks of gastroenteritis caused by uncultivable adenoviruses [Flewett et al., 1975; Richmond et al., 1979] and Ad40 in particular [Chiba et al., 1983].

There have been no studies described to date on the prevalence of subgroup F adenoviruses in rural Africa. In an attempt to determine the importance of these viruses, relative to rotavirus, in causing disease among children living under Third-World conditions, the present virological study was designed to coincide with a controlled bacteriological investigation being carried out at Shongwe Mission Hospital in KaNgwane, South Africa. All stools from this study were examined by sensitive and specific techniques for detecting subgroup F adenoviruses and rotaviruses. Electron microscopy was also used, as a safeguard against missing possible important etiological agents.

MATERIALS AND METHODS

Geographical Location and Study Population

The Nkomazi region of KaNgwane borders on Mozambique in the east, Swaziland in the south.

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The climate is subtropical with hot, wet, humid summers and cool, dry winters. The population lives in rural villages and derives its income from cattle or from shops and stalls. Many people work in the adjoining crop farming areas.

The socioeconomic status of the region is poor, and public services are minimal. There is no water-borne sewage system, no refuse removal, and an inadequate pure water supply. A large percentage of the population do not have latrine facilities and defecate in the fields. Overcrowding is prevalent in many homes, and this together with poor hygiene and contaminated water supplies contributes to a high prevalence of tuberculosis, gastroenteritis, and typhoid fever. The last described outbreak of cholera in South Africa occurred in this area in 1983 [Seedat, 1983].

The health requirements of the people are served by 19 outlying clinics and by Shongwe Hospital itself. The hospital has 218 registered beds, but averages about 400 inpatients at any one time. Each month, a large number of infants and young children suffering from acute diarrhea are admitted to the pediatric department. During the study period, February 1985 to January 1986, 1,738 infants were admitted to the infant ward, 557 (32%) of whom were suffering from diarrheal illness. Sixty-one (11%) of those with diarrhea died. The incidence of diarrheal disease is seasonal, most cases being admitted in summer.

Selection of Patients and Controls

Patients were selected for inclusion in the study if they had four or more liquid or semi-liquid stools per day. For the controls, infants matched for age (within 2 months) and time (within 5 days) who attended the outpatient department of the hospital or the outlying clinics were selected. Controls had no history of diarrhea (as defined above) within the previous 2 weeks.

Transport of Specimens

Stools were collected in 40 ml-capacity polystyrene containers. After removal of a sample for bacteriological analysis at Shongwe Hospital, the remainder of the specimens were sent by rail to Johannesburg. They were packaged with ice packs and usually reached the Virology Institute within 24 hours.

Electron Microscopy

Ten percent stool suspensions were made in distilled water, clarified to remove debris and bacteria, ultracentrifuged at 37,000 revolutions per minute (rpm) for 90 min in a Beckman SW 50.1 rotor, and prepared for electron microscopy by standard methods [Kidd et al., 1989]. Specimens were examined in a Jeol EX 1200 electron microscope at 80 kV and a magnification of $50,000\times$.

ELISA For Group A Rotaviruses

Ten percent stool suspensions were prepared in phosphate-buffered saline (PBS) and clarified at 3,000 rpm

(Sorvall rotor H1000B) for 30 min. Enzyme linked immunosorbent assay (ELISA) for rotavirus antigen was performed as described previously [Kidd et al., 1986] using 1:2 and 1:20 dilutions of clarified stool suspension.

Dot-Blot Hybridization Test for Ad40 and Ad41

Method B of Kidd et al. [1985] was used to extract DNA from 250 μ l of 10% stool suspension and to apply it to nitrocellulose paper. Briefly, after a standard sodium dodecyl sulfate (SDS), proteinase K, and phenol-chloroform extraction procedure to remove protein, the DNA was precipitated in ethanol (70% final) containing 0.3 M sodium acetate. After centrifugation and vacuum drying of the pellet, the DNA was resuspended in 250 μ l 10 mM Tris, 1 mM EDTA, pH 7.4. To render the DNA single stranded, 30 μ l of 1 M NaOH and 30 μ l of 3 M NaCl was added, and the mixture was placed at 95°C for 10 min, and then put on ice. Samples (40 μ l) were added to 100 μ l of 2 M ammonium acetate and applied by suction to nitrocellulose paper presoaked in 1 M ammonium acetate, using a Bio-Rad Bio-dot apparatus (Bio-Rad Laboratories, Richmond, CA). The paper was baked at 80°C for 2 hours. All samples were dotted on four separate papers, to be hybridized to Ad40-specific probe, Ad41-specific probe, a mixture of the two probes, and carrier plasmid (pBR322) DNA as control.

Nick-translated Ad40- and Ad41-specific probes, N26 and M9, and nick-translated pBR322 DNA, were used in standard overnight hybridizations at 65°C [Kidd et al., 1985]. The papers were postwashed extensively under conditions of low salt (0.015 M NaCl, 0.0015 M sodium citrate, 0.5% sodium dodecyl sulfate) at 65°C, then dried and autoradiographed, usually for 5 hours.

Infection of Chang Conjunctival Cells

Monolayer cultures of Chang cells were infected as described previously [Kidd and Madeley, 1981] except that 75 cm² flask cultures were used. Total DNA was extracted from the cells and digested with restriction enzymes by standard methods [Kidd, 1984]. For attempts to grow subgroup F adenoviruses in the presence of Ad2, 484 mm² sterile glass coverslips in six-well dishes (Nunc, Denmark) were seeded with Chang cells in Leibovitz L15 medium containing 10% fetal calf serum. After overnight incubation at 35°C, the medium was removed and replaced with 3 ml L15 medium containing 2% fetal calf serum and 4.4×10^5 fluorescent focus-forming units (FFU) of Ad2. Each stool extract was applied in 100 and 300 μ l amounts to adjacent wells, except for four wells that received only Ad2. After incubating for 44 hours at 35°C, the coverslips were washed in PBS and the culture fixed in acetone. They were treated for indirect immunofluorescence using a 1:500 dilution of mouse ascitic fluid containing subgroup F adenovirus-specific monoclonal antibody [Kidd and Blackburn, unpublished], followed by a 1:80 dilution of an FITC-conjugated rabbit antimouse im-

TABLE I. Results of Various Virus Detection Methods on Stools From 432 Rural Children

	Patients (n = 310)	Controls (n = 122)	Total (n = 432)
Electron microscopy			
Coronavirus-like	6	3	9
Rotavirus	21	3 ^a	24
Norwalk-like	1	0	1
Small round viruses	6	1	7
Adenovirus	5	0	5
ELISA			
Group A rotavirus	40	3	43
Dot-Blot			
Subgroup F adenovirus	41	3	44

^aOne of these specimens was negative by group A rotavirus ELISA. The results of EM and ELISA combined gave 44 positives.

munoglobulin serum (Nordic Immunochemicals, The Netherlands), as described previously [Tiemessen and Kidd, 1988].

To check the viability of the coinfecting virus, two of the Ad2 control coverslips were reacted with a 1:200 dilution of horse anti-Ad5 hexon group-specific antiserum (Wellcome Diagnostics, UK), followed by a 1:100 dilution of FITC-conjugated rabbit antihorse immunoglobulin serum (Nordic Immunochemicals, The Netherlands).

ELISA for Subgroup F Adenoviruses

Modification of the sandwich ELISA described by Kidd et al. [1986] was used. To render the test subgroup F adenovirus-specific, an undiluted hybridoma supernate fluid containing a subgroup F-specific monoclonal antibody (provided by Dr. Nalini, Singh-Naz, Children's Hospital, Washington, DC) was used as indicator antibody. This was followed by a peroxidase-conjugated rabbit antimouse immunoglobulin serum (Dako Laboratories, Denmark) diluted 1:200.

Intestinal Organ Culture

Cultures of intestinal segments from fetuses approximately 12 weeks old were prepared and maintained as described by Kidd and Madeley [1981], after Dolin et al. [1970]. On the day after preparation, the cultures were inoculated with 100 μ l stool suspension. Maintenance fluids were replaced every second day thereafter, and all fluids were stored at -20°C . For electron microscopy (EM) examination, each fluid (1.5 ml) was made up to 5 ml with distilled water and ultracentrifuged at 37,000 rpm for 90 min in a Beckman SW 50.1 rotor. The resulting pellets were resuspended in distilled water, stained with 2% potassium phosphotungstate, and examined by standard methods.

RESULTS

Between February 1985 and January 1986, 310 stool samples were taken from children with diarrhea at Shongwe Hospital, 98% of whom were aged 24 months

or less, and 79% aged 12 months or less. In addition, 202 control samples were taken, but because of the difficulties of having outpatient children defecate on demand, 80 of these samples were anal swabs and were not suitable for virological investigations. Thus, data from only 122 control subjects are included in the virological results.

Significant Environmental Data

The following data were derived from a full complement of 202 controls. More households of patients (11.8%) than of controls (3.5%) obtained drinking water from rivers ($P < .02$); more households of patients (33.5%) than of controls (13.1%) did not have latrines ($P < .001$); and more households of patients (26.3%) than of controls (11%) did not have adequate refuse disposal facilities ($P < .001$). These results indicate that conditions of poor hygiene and sanitation are predisposing factors for the development of acute diarrhea. A thorough study of the bacterial pathogens and viruses being shed by these children was therefore particularly relevant.

Relevant Bacteriological Data

Bacteriological results are to be published elsewhere [Wegerhoff et al., manuscript in preparation]. Briefly, enteropathogenic *E. coli* were the most frequently isolated pathogens, being recovered from 27.6% of patients and 32.2% of 202 controls. Enterotoxigenic *E. coli* were isolated significantly more frequently from patients (9.3%) than from controls (5.0%) ($P < .02$). *Salmonella* spp, *Shigella* spp, and *Campylobacter* spp were all recovered at rates below 6% in both patients and controls, whereas *Yersinia enterocolitica* was not isolated.

Electron Microscopy

Electron microscopy revealed that at least nine children shed coronavirus-like particles, similar to those seen in stools from Lesotho [Kidd et al., 1989]. Six children shedding these particles were patients and three were controls (Table I). All attempts to propagate coronavirus particles from four children in intestinal organ cultures failed, as detected by EM, although the cultures were maintained for between 11 and 17 days.

Rotavirus was detected by EM in 24 stool samples, 21 of which were from patients. Small, round, structured viruses were seen in seven stools, six of which were from patients. In addition, one stool from a patient had a virus resembling Norwalk agent [Kapikian et al., 1972; Kaplan et al., 1982]. Adenovirus particles were seen in only five stools, all of them patients (Table I).

Group A Rotavirus ELISA

Twenty-three of the 24 stools that were positive for rotavirus by EM were positive by ELISA, whereas one control stool was consistently negative by ELISA and may have contained a non-group A rotavirus (Table I). Thus, there was good one-way correlation between the

TABLE II. Results of Further Attempts to Analyse the 44 Subgroup F Adenovirus-Positive Stools Detected by Dot-Blot

	Patients (n = 310)	Controls (n = 122)	Total (n = 432)
Dot-blot	41	3	44
Subgroup F-specific			
ELISA	10	1	11
Culture	3	0	3

results of EM and ELISA, but with ELISA detecting 70% more positives. However, the ELISA positives that were negative by EM had low optical density values (a ratio of 2.5 times the preimmune control for each specimen was considered to be positive). This would explain the failure to detect these positives by EM. Another explanation is that particles may have been broken up during shipment and storage and could therefore not be identified by EM but could be detected by ELISA.

In total, ELISA for group A rotavirus showed 43 positive stool samples, 40 of which were from patients and three of which were from controls. The ages of the infected children ranged between 2 months and 2 years. Thus, significantly more patients (12.9%) than controls (2.5%) shed group A rotavirus ($P < .01$). For 28 of the 40 rotavirus-positive patients (70.0%), rotavirus was the sole recognizable cause of diarrhea. Nine percent of patients and 2.5% of controls shed group A rotavirus and no other pathogen, the difference still being statistically significant ($P < .05$).

Dot-Blot Hybridization for Ad40 and Ad41

DNA hybridization using the Ad40- and Ad41-specific probes revealed that 44 children shed subgroup F adenoviruses (Table I). Although there are some cross-reactions between the two serotypes with these probes, examination of the relative intensities of the dots on autoradiographs revealed that 34 strains were Ad40 and ten strains were Ad41. All of the ten Ad41-positive stools and 31 of the 34 Ad40 strains were from patients, whose ages ranged between 3 weeks and 2 years. The proportion of patients shedding subgroup F adenoviruses was 13.2%, and the proportion of controls was 2.5%. Thus, on the basis of the dot-blot results, significantly more patients than controls shed subgroup F adenoviruses ($P < .001$). Of the 44 subgroup F adenovirus positive stools, three also contained rotavirus. For 19 of the 41 subgroup F adenovirus-positive patients (46.3%), these were the sole recognizable pathogens detected in the stool. More patients (6.1%) than controls (1.6%) shed subgroup F adenoviruses alone ($P < .1$).

The results of dot-blot hybridization did not correlate well with the results of EM. Of the five adenovirus-positive stools by EM, three were dot-blot positive and two were negative. The latter two samples were proven to contain serotypes other than Ad40 and Ad41 by culture. However, because of the large discrepancy apparent between the results of EM and dot-blot, attempts

were made to confirm the positive dot-blot results by culture and ELISA using monoclonal antibodies.

Attempts to Culture Subgroup F Adenoviruses

The 44 dot-blot positive stool suspensions were used to inoculate Chang conjunctival cells, and the cultures were monitored for cytopathic effects every 2 days. Only three samples gave positive results (Table II), which were confirmed by extraction of the DNA followed by digestion with restriction endonuclease Sma I. One strain proved to be Ad40 and the other two were Ad41, which correlated with the dot-blot results.

Attempts to Reactivate Subgroup F Adenoviruses

All stools that were positive for adenoviruses by EM thus yielded adenoviruses in culture. However, the positive dot-blot results for 41 stools were not yet confirmed, and attempts to culture the viruses were continued using a possible helper virus. We have shown recently that Ad41 growth is enhanced in the presence of coinfecting Ad2 in Chang cells and is solely dependent on the presence of Ad2 in human embryonic fibroblasts [Tiemessen and Kidd, 1988]. It was possible that the growth of defective subgroup F adenoviruses in stool from this study might be complemented by addition of Ad2 at the time of inoculation. However, Chang cells inoculated with any of the 41 stool extracts and Ad2 simultaneously did not show subgroup F adenovirus late antigen synthesis, as detected by immunofluorescence using a subgroup F-specific monoclonal antibody.

ELISA for Subgroup F Adenovirus

In a final attempt to substantiate the positivity of the 44 dot-blot samples, they were tested by ELISA using a subgroup F-specific monoclonal antibody [Singh-Naz and Naz, 1986]. Eleven of the 44 stools, including the three culture-positives, were detected by this technique (Table II). Ten of the 11 ELISA positives were from patients. Thus, eight further dot-blot positives were confirmed, and it was clear that these eight antigen-positive stools had lost viability. The other 33 dot-blot subgroup F positives remained unconfirmed by any other test.

Seasonal Shedding of Viruses

Eight of the nine coronavirus-positive stool samples had been taken within 5 weeks of each other (in February–March, 1985), whereas the ninth was taken in November 1985, and this was the most obvious example of seasonal shedding (Fig. 1). Rotavirus (as detected by ELISA and/or EM) was shed throughout the year, with a notable increase in winter from May to August, accounting for 61.4% of the 44 positives. Subgroup F adenoviruses (as detected by dot-blot) were shed throughout the year, with an increase in summer between late-September and late-January, accounting for 63.6% of the 44 positives.

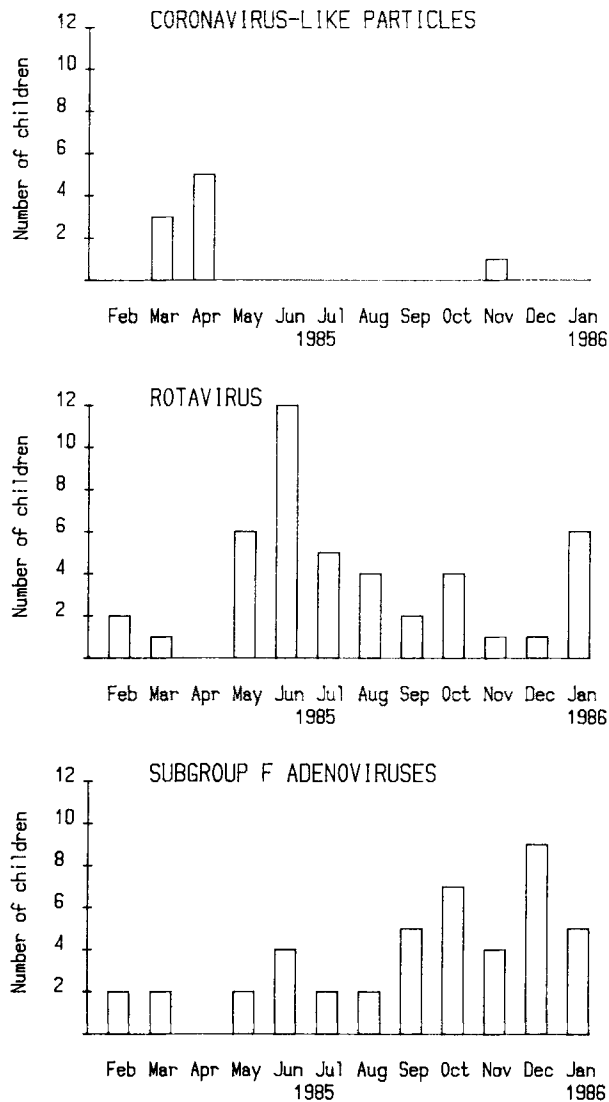


Fig. 1. Seasonal shedding of coronavirus-like particles, rotavirus, and subgroup F adenoviruses.

DISCUSSION

This study was an attempt to determine the degree of association between acute diarrhea of infants in a rural African setting and the shedding of subgroup F adenoviruses and to compare the prevalence of shedding of these viruses to that of rotavirus and other viral agents.

The proportion of patients and controls shedding rotavirus compares well with results of similar studies in developing countries [Fagbami et al., 1985; Sitbon et al., 1985; Bhan et al., 1988; Steele et al., 1986a]. The peak of rotavirus shedding in winter agrees with the results of a 3 year study carried out in another rural area of Southern Africa [Steele et al., 1986b]. A similar peak in rotavirus shedding was noted during a 1-year study of hospitalized children from Soweto [Kidd et al.,

1986], except that the peak in the latter study occurred earlier in the year (March–April).

The finding of coronavirus-like particles in the stools of children living in a Third-World environment might have been predicted from our earlier studies [Kidd et al., 1989] and those of others in Africa [Sitbon, 1985]. It is by no means clear if these particles have a role in causation of diarrhea, and their viral nature has been questioned [Dourmashkin et al., 1980; Schnagl et al., 1987]. Our attempts to culture these agents, using techniques similar to those said to give positive results by others [Resta et al., 1985; Caul and Egglestone, 1977], failed. This may have been due to inadequate precautions in maintaining low temperatures during transport to the virology laboratory.

The most surprising result of this study is the high proportion of stools from patients who scored positive for subgroup F adenovirus by dot-blot hybridization (13.2%). This figure is almost twice that reported by Uhnoo et al. [1984] for a controlled study in Sweden, where the viruses were detected by EM, culture, and ELISA. If only patients are considered for whom subgroup F adenoviruses were the only potential pathogen detected in our study, the infection rate is then 6.1%, which is comparable to the figure of 7.2% found by Uhnoo et al. [1984].

That only five specimens of 310 from patients (1.6%) were positive for adenoviruses by EM is unusual. Other workers have reported figures of 5–15% [Gary et al., 1979; Retter et al., 1979; Brandt et al., 1985]. Since only adenoviruses seen by EM in our study could be cultured, and none of the other ELISA positives showed evidence of viability, there is a possibility that the structural integrity of particles in the majority of cases did not survive shipment and storage. Another explanation relates to our recent finding that infection of Chang cells by Ad41 follows multiple-hit kinetics [Tiemessen and Kidd, in preparation]. Thus, successful infection requires a certain minimum virus concentration, and a dilute inoculum is not likely to cause cytopathic effects. This minimum required concentration may not have been achieved in stools, perhaps due to a combination of low virus output and adverse storage conditions. If this was the case, it is clear that the presence of another adenovirus, known to aid Ad41 replication normally [Tiemessen and Kidd, 1988], did not improve infectivity.

The ELISA test using monoclonal antibodies confirmed the results of dot-blot hybridization in 11 cases, but the majority of the dot-blot positives remained unconfirmed. Hybridization is a relatively sensitive technique, being able to detect 10 pg or less of homologous viral DNA under ideal conditions [Kidd et al., 1985]. Adenoviruses, in general, are known to produce an excess of structural protein components, which would be detected in stool by ELISA, but it is difficult to determine how much virus DNA (cell associated or particle associated) would be produced in stool relative to the structural proteins. Unfortunately, hybridization

stands alone at the moment as a means of detecting adenovirus DNA in specimens, and there is no other technique with which to compare it. The technique of DNA amplification [Saiki et al., 1985] is being applied to many areas of virology and may help answer some of these questions.

On the basis of the dot-blot results for subgroup F adenoviruses, the proportion of patients and controls shedding these adenoviruses was very similar to the proportion shedding rotavirus as detected by ELISA. Eighty-two children shed one or the other virus, and only three children shed both. Rotavirus was found to be the sole recognizable pathogen in a higher proportion of patients than were subgroup F adenoviruses. However, the peak of rotavirus shedding was in winter, whereas the peak of subgroup F adenovirus shedding was in summer, when bacterial infections are most common [Kahn, 1957; Freiman et al., 1977]. Since the difference in subgroup F adenovirus shedding between patients and controls was statistically significant and markedly similar to the figures for rotavirus, the subgroup F adenoviruses appear to be an important cause of diarrhea in this rural African environment.

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