Isolation and Identification of Adenovirus Recovered from the Stool of Children with Diarrhoea in Lagos, Nigeria

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SUMMARY

In order to establish the role of adenovirus in gastroenteritis in Nigerian children, stool samples were collected from 138 young children with gastroenteritis and 29 other age-matched controls. The samples were inoculated into 6 different tissue culture cell lines and isolates with characteristic CPE were subjected to CFT confirmation of the presence of adenovirus antigen. All the samples were screened for adenovirus by a commercially available enzyme immunoassay (Biotrin Adenovirus Antigen EIA) for the presence of the group antigen. Of the 138 stool samples from children with diarrhoea screened by EIA, on 23 (16.7%) were positive, while 4(13.8%) of the 29 controls were also found positive. A greater proportion of the adenovirus-positive cases were aged between 13 and 24 months. There was no difference in the prevalence of the infection between male and female. The fastidious, enteric adenoviruses of subgroup F were sought utilizing a second EIA (AdenoClone), and occurred in 3.6% of the samples from diarrhoeic children and was not detected in the control group. There was no significant difference between the clinical symptoms of children infected with adenovirus and those not infected with adenovirus. However, the source of drinking water had a significant effect on the frequency of stool per day. The infection occurred all year round except for April and there was no significant correlation with the climatic factors. This study implies that the fastidious adenovirus is important in the aetiology of diarrhoeal illness in Nigerian children.

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Introduction

Human adenoviruses are associated with a wide range of clinical diseases including respiratory, ocular and gastrointestinal tract infections. Particular serotypes of adenovirus are associated with various clinical conditions [1]. The adenoviruses most frequently associated with gastroenteritis are those of subgroups A (types 12, 18 and 31) and F (types 40 and 41) and these strains of subgroups A and F are therefore referred to as enteric adenoviruses (Eads) [2]. In addition, the subgroup F adenoviruses are referred to as fastidious enteric adenoviruses, because they are not readily cultivatable in routine tissue culture [3].

The enteric adenoviruses, specifically those belonging to subgroup F, have been reported in many regions of the world where they are associated with acute diarrhoeal illness in young children [3-7]. The subgroup F Eads have been recorded as the second most important cause of acute infantile gastroenteritis after rotavirus [5-8]. Enteric adenoviruses have also been found to be a common enteric virus associated with diarrhoea in Africa, although limited data area available [9,10]. In many of these studies, routine electron microscopy was utilized, although enzyme linked immunoassays are now commercially available.

There have been few studies in Nigeria or West Africa looking for the incidence and occurrence of the fastidious enteric adenoviruses. In 1992, Avery *et al* did not detect any adenovirus from 66 diarrhoeal stool specimens examined using EM [11]. Olaleye *et al* reported that serological evidence indicated that adenoviruses are important agents of acute respiratory infection in Nigerian children [12]. More recently, adenovirus was determined to be present in 3.8% of the stools of a small cohort of diarrhoeal children in Jos [13].

In this more extensive study, investigating diarrhoeal disease in young children in Lagos, Nigeria we included assays for the determination of the relative importance of the fastidious enteric adenoviruses (subgroup F) in the role of gastroenteritis in Nigerian children.

Materials and Methods

Patient samples

This was a hospital-based, cross-sectional study and included children attending the Lagos University Teaching Hospital. A single stool sample was collected from 138 children under 5 years old who presented to the Children's Emergency Ward with diarrhoea. Diarrhoea was defined as the passage of three or more watery stools within the last 24-hour period. A further 29 stool samples were collected from the Immunization Clinic at the Hospital and served as an age-matched control group. None of these infants had had a history of diarrhoea in the previous two weeks.

Questionnaires were used to obtain information from the parents or guardians accompanying the child to hospital. Information included signs and symptoms of illness (abdominal cramps, diarrhoea and vomiting, respiratory symptoms, elevated temperature etc). Every child was examined for dehydration using the WHO standard criteria [14]. Information was also obtained from the parent/guardian on housing conditions and drinking water supplies for the household. Treatment of the water before consumption was also included. Hospital records were accessed after the collection of the stools to investigate the admission status of the child, duration of diarrhoea and severity of the diarrhoeal episode.

The stool samples were frozen at -20^{0}C after collection and before analysis. The frozen stool samples were thawed once and 0.1g of stool was added to 0.9ml phosphate buffered saline (PBS), supplemented with penicillin and streptomycin, to give 10% w/v. This mixture was then centrifuged at 2300g for 30 min and the clarified supernatant fluid was inoculated immediately onto tissue culture.

Tissue Culture Isolation

HEP-2, Vero, B95a, BHK, Hela and L20B cell lines were used for viral isolation. The cells were grown on Eagles Minimum Essential Medium (MEM) containing 10% foetal calf serum (FCS) and maintained in MEM containing 2% FCS supplemented with 200 units/ml of penicillin and 200ug/ml of streptomycin. The clarified stool supernatant was inoculated into the monolayer cells. All cultures were incubated at 37^{0}C and readings for cytopathic effect (CPE) were made daily for 7 days or up to 14 days for those with no obvious CPE. The cells showing CPE of about 70-90% were harvested and stored away at -20^{0}C. Samples were treated to three cycles of freeze-thawing of the harvested cells in a 37^{0}C water bath to increase the virus yield by dissociating the virus from the cells.

Complement Fixation Assay

The tissue culture isolates were examined by the complement fixation test procedure for confirmation of the presence of adenovirus antigen, using mirotitre techniques described previously [15]. Commercially prepared adenovirus antisera (Serotec, USA) was used. Briefly, equal volumes (25ul) of veronal buffer and test antigen from infected cells were mixed in microtitre plates and incubated for 30 min at 56^{0}C. This was then left at room temperature for 90 min to cool. Equal volumes

(25ul) of each of the commercially prepared adenovirus antisera and guinea pig complement were added and incubated for 37^{0}C for 90 min. Finally 100ul of sensitized sheep red blood cells were added and incubated for a further 90 min at 37^{0}C. Lysis of the cells indicated the absence of adenovirus antigens, while a red pellet indicated the presence of the antigen.

Adenovirus Antigen Enzyme Immunoassay (EIA)
A commercial ELISA kit (Biotrin Adenovirus Antigen EIA, Biotrin International) was used for the detection of group adenovirus antigen in 10% suspension faecal specimens. The test utilizes specific adenovirus antibody to capture the antigen present in stool and a genus-specific hexon antigen, common to all adenovirus serotypes, for the detection. The test was performed as specified by the manufacturers and the results were ready spectrophotometrically at 450nm. In brief, 10% faecal suspensions were introduced into each well of the coated microtitre plate and incubated at ambient temperature with anti-adenovirus conjugate for 60 min. After washing, the substrate was added and incubated for 15 min at ambient temperature before adding the stopping solution. The generation of a blue colour indicated the presence of adenovirus antigen in the stool.

AdenoClone (40 and 41) EIA
A second commercial assay (AdenoClone, Cambridge Biotech) was utilized to screen the adenovirus positive stools from the Biotrin assay. The second ELISA is based on monoclonal antibodies specific for the subgroup F adenoviruses (types 40 and 41). The assay was performed as specified by the manufacturer's specifications.

Climatic Factors
The information on climatic factors was obtained from meteorology division of the Federal Ministry of Aviation, Oshodi.

Statistical Analysis
The Epi Info software version 6.0 was used. The Fig. P Programme and logistic regression Programme of Dallal [16] were also used.

Results

Tissue culture
Cell damage was noted in at least one of the cell lines in 85 of the 138 specimens obtained from diarrhoeal specimens. However, only 13 (15%) out of the 85 stool samples inoculated into tissue culture cells showed characteristic cytopathic effect (CPE) in the form of grape-like clusters of round, refractile enlarged cells. These 85 specimens were analyzed by a complement fixation assay to confirm the presence of adenovirus antigen. In addition, a further 3 specimens, which were found to be negative by tissue culture, were found to be positive by enzyme immunoassay (below).

Complement Fixation Assay
The CFI assay was performed on the tissue culture supernatants of the 85 specimens showing some kind of cell damage. However, only 11 (15%) were positive by this assay. It was noted that the children with a positive result were slightly older than those that were negative for adenovirus. In addition, older children had higher titres of adenovirus antigen than the younger age group even though the titres were generally low.

Adenovirus Antigen ELISAs
Of the 138 stool samples screened for a common adenovirus antigen by the Biotrin EIA, only 23 (17%) were positive for adenovirus antigen, while a further 4 (14%) of the controls were also positive (Table 1). All the CFT positive specimens were confirmed to be positive by EIA, indicating the accuracy of the assay. A greater proportion (28%) of the positive cases was between 13 and 24 months of age for the diarrhoeal cases, although there was no particular age distribution pattern. While 18% (14/78) of males with diarrhoea were infected with adenovirus, 15% (9/60) of females with diarrhoea were also infected.

Thus, there was no significant difference in the infection between both sexes.

There was no statistical difference in the clinical symptoms between diarrhoeic children infected with adenovirus and those not infected with adenovirus. However, it appeared that adenovirus infected children seemed to have increased numbers of cases of dehydration, abdominal pain, respiratory symptoms with longer frequency of stool per day and a higher rate of admission (Table 2). Using a logistic regression Programme, adenovirus infection was not significantly associated with any of the clinical symptoms and none of these symptoms was prognostic for hospitalization. However, the source of drinking water had a significant effect ($P<0.05$) on the frequency of stools passed per day.

Adenovirus infection occurred all year round except for the month of April and its highest prevalence was in February and March. The isolation of adenovirus was not significantly correlated with any climatic factors though temperatures and vapour pressure tended to be more positively correlated.

Table 1: Age distribution of adenovirus isolates among children in Lagos State.

Age (m)	Diarrhoea Group			Control Group		
	Number	Positive	%	Number	Positive	%
0-5	47	3	6.4	24	3	12.5
6-12	60	12	20.0	3	1	33.3
13-24	18	5	27.8	0	0	0
>25	13	3	23.1	2	0	0
Mean age	9.9m	12.8m		5.1m	6.4m	
Total	138	23	16.7	29	4	13.8

Table 2. Clinical features associated with adenovirus infection in some children in Lagos State

Clinical Features	Adenovirus Infected children (n=22)	Non-infected Children (n=11)	P-value	X2
Fever	15(68.2)	77(69.4)	>0.05	0.01
Vomiting	14(63.6)	71(64.0)	> 0.05	0
Dehydration	13(59.1)	47(42.3)	>0.05	2.08
Abdominal pain	4(18.2)	17(15.3)	>0.05	0.03
Respiratory symptoms	6(27.3)	23(20.7)	>0.05	0.26
Hospitalized	17(77.3)	74(66.7)	>0.05	0.96
*Mean length of stay	2.6d	2.7d	>0.05	
*Mean frequency of stools per day	5.6	5.3	>0.05	
*Mean duration of diarrhoea	4.6d	5.5d	>0.05	

Fastidious Enteric Adenoviruses

When the adenovirus positive specimens from the previous assays were collated (27 in total) and analyzed by the specific subgroup F assay, only five specimens were determined to be adenovirus types 40 and 41. (The assay does not differentiate between type 40 and 41). These strains were all in the diarrhoea group and none occurred amongst the four adenovirus strains recovered from the control group. Thus the typical enteric adenoviruses of subgroup F are associated with 3% (4/138) of the total number of diarrhoeal episodes seen. The subgroup F adenovirus serotypes constituted 22% of the total numbers of adenoviruses detected in the diarrhoeal stools of young children with gastroenteritis.

Discussion

Overall, adenoviruses were detected in 16.7% of the diarrhoeal stools of the infants and young children with diarrhoea and in 13.8% among the small control group included in this study. The similarity of the prevalence amongst the two groups probably reflects the fact that adenoviruses of serotypes, other than the classical enteric adenoviruses of subgroup A and F, are often excreted in the stool [2 ,4, 17]. This is further confirmed by the fact that no subgroup F adenoviruses were detected in the control group with a more specific assay for types 40 and 41.

The epidemiological surveillance of the enteric adenoviruses (subgroup A and F) is restricted by the limited assays available for their detection. In addition, the costs of the molecular typing assays, such as restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR), prohibit their use in the routine diagnostic laboratory. Thus studies to investigate the epidemiology and aetiology of the adenoviruses in diarrhoeal stools are based on utilizing the fastidious nature of the enteric adenoviruses and the available monoclonal antibodies - such as included in AdenoClone. A major limitation of this, and other studies in Africa, therefore includes the failure of utilizing the appropriate assays for the subgroup A adenoviruses which have been included in diarrhoeal episodes.

This study, however, does indicate the importance of the subgroup F adenoviruses in the aetiology of diarrhoea among young Nigerian children attending hospital for the severity of the diarrhoeal episode. The prevalence of infection in this study (3%) is similar to the 3.8% reported in the Plateau State of Nigeria in a study also utilizing an enzyme-linked assay for the detection of enteric adenoviruses [13]. Although limited, this study also shows that the strains circulate year round and that the virus types 40 and 41 seem to infect slightly older children. This is the first report of the detailed epidemiology of enteric adenoviruses in Nigeria, and supports the earlier, smaller studies that adenoviruses should be included in further epidemiological studies investigating diarrhoeal disease.

It is not surprising that adenoviruses of other serotypes were found in relatively high prevalence in the control group because they cause a number of other symptoms including respiratory symptoms, conjuctivitis, and pneumonia that would all be common in infants [18]. However, this high proportion of other serotypes of adenoviruses in controls could be misleading since they could be excreted without symptoms for at least 2 years after primary infection [18].

Although there was a tendency for the infection to be more common among children 13 to 24 months of age, there was no statistically significant age distribution pattern. Akhter *et al,* [19] and Yamashita *et al* [17] reported that adenoviruses usually infect children up to the age of 3 years, but primarily 2 years old and below are more prone to its infection. Furthermore, the seasonal distribution of enteric adenovirus infection has been shown in several studies [6, 8, 10]. However, the shedding in stool of all adenovirus serotypes has not been reported and it is not surprising, with the different clinical conditions associated with the family, that adenovirus would be shed year round. The numbers of enteric adenovirus 40/41 in this study do not enable a proper evaluation of their seasonal role in Nigeria.

Christensen [20] concluded after a review of several studies that adenovirus infected

patients have milder diarrhoeal disease when compared to the non-adenovirus infected patients, however in this study there was no significant difference between the two groups. It was also found that there was no clinical symptom that was significantly associated with adenovirus infection neither was there any that was prognostic for hospitalization. However, the source of drinking water had a significant effect on the frequency of stools per day and the duration of diarrhoea. The mean duration of diarrhoea of 4.6 days in this study, is shorter than those reportedly caused by fastidious adenoviruses [8, 17] and Uhnoo *et al*, confirmed that non-fastidious adenovirus was responsible for shorter duration of diarrhoea [6].

This study indicates that the fastidious adenoviruses are important causative agents of diarrhoea in Nigerian children as opposed to the other serotypes, which were found to be endemic in both diarrhoeal and control cases. Since the source of drinking water was found to have significant effect on the frequency of stool per day in diarrhoeic children, it is therefore important that clean and treated water gets to the populace which will alleviate the burden of diarrhoea among the Nigerian children [21].

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