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An epidemic of acute diarrhoea in rural southern India associated with echovirus type 11 infection

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SUMMARY

An epidemic of diarrhoea with two distinct waves affected a village of 1375 people in southern India in 1983. The first wave of the epidemic, from the last week of December 1982, had a sharp peak in January 1983 and was over by March. Echovirus type 11 was isolated from patients, who also had a serum antibody response to the virus. During the second wave of the epidemic, from May to September 1983, the clinical features were different and *Shigella flexneri* was isolated without significant viral isolates. Infection during the first wave did not protect from the second wave. Virus isolation was in human intestinal tumour-derived differentiated epithelial cell lines; such cell lines may be useful for the isolation and identification of enteroviruses in clinical samples.

INTRODUCTION

In rural southern India, in addition to endemic acute diarrhoea, epidemics of diarrhoea affecting villages occur from time to time. Epidemics of diarrhoea due to tropical sprue and to bacterial and viral infections have been reported earlier from the rural areas surrounding Vellore (Mathan & Baker, 1968, 1971; Mathan, Kurian & Mathan, 1982*a*; Mathan *et al.* 1982*b*, 1984; Kapadia *et al.* 1984). Although a variety of bacterial and viral agents have been shown to have a causal relationship to acute diarrhoea, enteroviruses are, in general, considered not an important cause (Wenner, 1972). However, there are isolated reports of enterovirus-associated diarrhoea in institutional outbreaks (Yow *et al.* 1970) or in immuno-compromised individuals (Yolken *et al.* 1982). This paper reports an epidemic outbreak of diarrhoea in a village 20 km east of Vellore between December 1982 and October 1983. Two distinct waves of the epidemic were identified. The first was associated with an echovirus type 11 infection which was not present during the second wave of the epidemic.

SUBJECTS STUDIED AND METHODS

The village and its population

Punganoor, an agricultural village 20 km to the east of Vellore, has a population of 1375. The village is a cluster of houses arranged around streets occupying a

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total area of less than 0·5 km^2 surrounded by fields where most of the adults work. Rice, sugar-cane, groundnuts and vegetables for local consumption are grown in these fields, owned by a few of the rich families in the village. There is a school in the village up to the fifth grade and children go to a high school 3 km away. The nearest town with medical facilities is 10 km away. Drinking water is obtained from three deep-bore wells in addition to a large number of shallow surface wells located in the backyards of houses. About half the houses have plastered brick walls and tiled roofs while the rest have mud walls and palm leaf or straw thatch. There were no sewage or garbage disposal arrangements and the yards and surrounding fields were used for defecation. The villagers are vegetarians, rice being the staple diet, supplemented with other locally grown cereals, vegetables and pulses. Due to economic constraints, meat and fish are seldom consumed.

Epidemiological methods

The first report of the epidemic was received during the first week of January 1983. The population was enumerated and all cases of diarrhoea were identified. Symptomatic therapy and advice regarding adequate intake of oral fluids was provided at twice-weekly clinics. Every family in the village was visited on alternate days to record new cases of diarrhoea, for follow-up and to ensure that patients took oral fluids. Patients who were willing to go into hospital were admitted to a metabolic ward.

Laboratory methods

Stool samples. Stool samples were collected from patients and matched controls on three occasions: 18 January, 3, 18 and 25 February and 5–11 July. Controls were defined as individuals in the same or an adjacent family to the patient who gave no history of diarrhoea for the preceding 4 weeks and who were within 1 year of age of the patient in children and 5 years in adults. Although an attempt was made to collect at least one control stool sample for each patient, this was not always possible due to non-compliance. All stool samples were passed directly into 2 in diameter sterile plastic containers and were transported, chilled on ice, to the laboratory within 2 h. On 18 January stools were obtained from 8 controls (2 of whom developed diarrhoea the next day) and 18 patients with diarrhoea (1 day to 5 weeks' duration). On 3, 18 and 25 February stools were obtained from 10 controls, 14 patients who had recovered from diarrhoea and 19 patients who still had diarrhoea (6 days to 8 weeks duration). During July 1983, 10 families totalling 49 individuals were selected with at least 1 patient with diarrhoea of less than 5 days' duration in each family. Stools were obtained from 39 individuals (13 with diarrhoea and 26 controls) on 5, 7, 9 and 11 July. From 9 individuals 3 samples each, from 13 individuals 2 samples each and from 17 individuals single stool samples were obtained.

Virus isolations. Approximately 20% (w/v) suspensions of stools were prepared in ice-cold RPMI-1640 (Flow Laboratories, Irvine, U.K.) with 5% bovine serum albumin fraction V (Sigma London Chemical Co. Ltd, Poole, U.K.), 100 units of penicillin and 100 μg streptomycin per ml (RPMI-BSA). The suspensions were clarified twice by centrifugation at 10000 g for 1 h and the supernatant was divided into aliquots, several of which were frozen while one was inoculated into

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tissue cultures. Three different human intestinal tumour-derived differentiated continuous epithelial cell lines were used for virus isolations. These were HRT-18 from rectal carcinoma (Tompkins *et al.* 1974) (kindly supplied by Dr J. Laporte, INRA, Theiverval-Grignon, France) and SKCO-1 and HT-29 derived from human colonic cancers (Fogh & Trempe, 1975) (The Memorial Sloan Kettering Institute, New York).

Cell cultures were grown as monolayers, either in glass medical flat bottles (stock cultures) or in 16×120 mm stoppered glass tissue culture tubes (virus isolation) at 37 °C using media, including fetal calf serum (FCS), obtained from Flow Laboratories, Irvine, U.K. HRT-18 and HT-29 cell lines were grown in RPMI-1640 medium supplemented with 10% FCS, 15 mM sodium bicarbonate and antibiotics. SKCO-1 cell line was grown in Dulbecco's modification of Eagle's minimal essential medium (DMEM) supplemented with 15% FCS, 1% amino acid supplement, 15 mM bicarbonate and antibiotics. Confluent monolayers were subcultured at a ratio of 1 to 2 (SKCO-1) or 1 to 3 (HRT-18 and HT-29) every 4–5 days, using trypsin 1 in 250,(DIFCO Laboratories, Detroit, U.S.A.) (0·07%) and disodium versene (0·03%) mixture in Dulbecco's phosphate buffered saline A (PBSA) (pH 7·3).

Confluent monolayers obtained 4 days after seeding about 2×10^5 cells in 1 ml medium per tissue culture tube were washed with PBSA and inoculated with 0·1 ml of freshly prepared stool suspension which was allowed to adsorb for 1 h at 37 °C. Each monolayer was then washed and incubated at 37 °C with Leibovitz L15 medium containing 2% FCS and antibiotics (L-15-2). Cultures were examined daily for cytopathic effect (CPE) and frozen at −70 °C for further passage after 3 days. Two further passages were made as described above. Monolayers inoculated with RPMI-BSA were included as controls.

Virus identification. About 2×10^4 SKCO-1 cells suspended in 0·1 ml L-15 medium supplemented with 15% FCS, antibiotics and 1% Flow's amino acids were added to each well of 96 well microtitre plates (Sterilin Ltd, Teddington, U.K.). Sealed plates were incubated at 37 °C in a humidified incubator for 3 days to obtain confluent monolayers. About 200 $TCID_{50}$ of third-passage isolates were identified by a neutralization test using LBM intersecting serum pools (Lim & Benyesh-Melnick, 1960; Schmidt *et al.* 1971) as described elsewhere (Patel *et al.* 1984).

Virus neutralizing antibody in serum. Titres of neutralizing antibodies to echovirus 11 in 16 serum samples from asymptomatic controls, patients with diarrhoea and those who had recovered during January and February 1983, were determined using a Colindale echovirus 11 reference strain (kindly supplied by Dr M. Roebuck). A constant amount of virus was incubated at 37 °C for 2 h with serial twofold dilutions of test sera and 20 μl of each mixture was then inoculated on to monolayers of SKCO-1 cells using two wells per mixture. Virus dose was found to be about 200 $TCID_{50}$ in each test upon titration of virus-diluent mixture after a similar treatment as the test mixtures. The reciprocal of the serum dilution completely neutralizing challenge virus was read 3 days after inoculation.

Electron-microscopic examination of faecal samples. All stool samples from controls and patients were prepared for direct electron microscopy. After clarification of a 20% suspension of faeces in phosphate-buffered saline, pH 7·0 at 10000 ***g*** for 10 min the supernatant was centrifuged at 90000 ***g*** for 90 min and the pellet from this step examined after negative staining.

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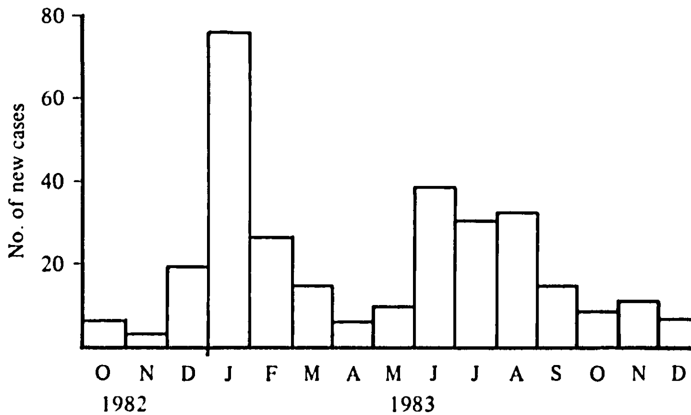

Fig. 1. The epidemic curve. Number of new cases of diarrhoea each month from October 1982 to December 1983.

Table 1. *Attack rates per hundred in different age groups in the two waves of the epidemic*

Age group (years)	No.	Attack rates Dec–Apr.*	Attack rates May–Nov., fresh cases†	Attack rates May–Nov., secondary cases‡
< 5	200	22·5	27·7	55·5
5–< 12	281	4·9	8·2	42·8
12–< 18	284	3·2	5·4	11·1
> 18	1109	6·3	8·5	15·7

* Attack rates per hundred calculated for total population, December–April.
† Attack rates calculated excluding those affected December–April.
‡ Attack rates calculated for those who initially developed diarrhoea December–April, recovered and then affected again May–November.

Bacteriological studies. All stool samples obtained from the village were cultured in standard media for isolation of bacterial enteric pathogens (Rajan & Mathan, 1982).

Clinical studies. Thirty-three patients (19 in January–February and 14 in June–August) were admitted from the village into a metabolic ward. Tests of intestinal absorption were carried out on these patients by previously described methods (Baker & Mathan, 1971).

RESULTS

The epidemic

The epidemic started in this village in the last week of December 1982. By December there were 21 patients in the village, of whom 10 were children below the age of 5 years. The highest number of new cases (77) occurred in January 1983 (Fig. 1). New cases of diarrhoea decreased in February and March and by the middle of March the epidemic appeared to be over. In April there were only six new cases of diarrhoea in the village, which is the expected background of endemic

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Table 2. *Enterovirus isolations from patients and controls, July* 1983

Identification no.	Age (years)	Date of collection: 5 July	7 July	9 July	11 July
Patients					
61–5	14	—	E7	N	E30
64–4	22	N	—	P2	—
64–7	15	N	—	U	N
240–3	25	B6	—	—	—
272–3	2	B6	—	—	—
282–1	65	N	—	U	U
282–4	3	U	—	—	U
288–3	3	—	N	—	U
315–1	17	—	N	—	E30
4 patients		N(2)	N(2)	N(2)	N(2)
Controls					
58–5	8	—	E7	—	N
58–8	12	—	E7	—	U
58–9	10	—	E7	N	U
58–10	1	—	E7	—	U
58–15	65	—	E7	N	U
61–2	35	—	N	P2	P2
61–4	15	—	—	—	U
64–5	1	P2	—	P2	—
266–5	6	—	E30	—	—
282–2	28	N	—	P2	N
282–3	24	U	—	P2	P2
288–1	30	—	—	U	—
315–6	10	—	E30	N	U
13 controls		N(3)	N(5)	N(5)	N(6)

Identification numbers are family numbers followed by individual's number in family.—, No specimen obtained; E7, echo type 7; E30, echo type 30; N, no isolate; P2, polio type 2; U unidentified isolate; B6 coxsackie B6; In patients and controls without any isolates the number of negative specimens each day is indicated in parentheses.

acute diarrhoea. From the latter half of May new cases of diarrhoea again appeared with increased frequency (Fig. 1). There was no sharp peak, but during June, July and August more than 30 new cases occurred each month.

During each wave of the epidemic, new cases were found in all parts of the village. No particular association with any area of the village, source of drinking water or any other common factor could be found. All age groups were affected (Table 1). The attack rate in children was significantly higher during both waves of the epidemic. Twenty-eight individuals who had recovered from diarrhoea during the December-to-March period again developed diarrhoea during May to November. This second attack of diarrhoea occurred after asymptomatic periods ranging from 1 month to 4 months. The attack rate in this group was significantly higher than among those without diarrhoea during December–March (Table 1).

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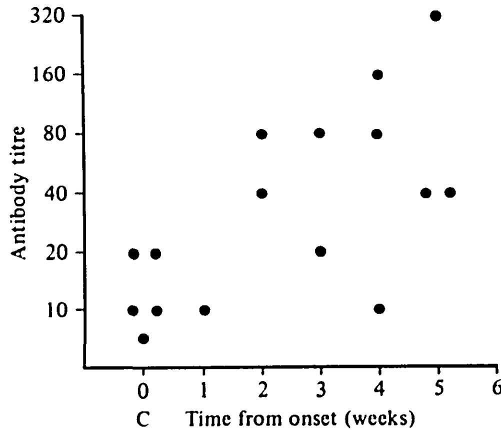

Fig. 2. Reciprocal of neutralizing antibody titres to echovrius type 11 in serum obtained from controls (C) and patients at different periods from the onset of diarrhoea (0).

Virological studies

Fifteen isolates of echovirus type 11 were obtained from the 18 patients on 18 January 1983. Echovirus type 11 was isolated from 2 of the 8 controls, 2- and 5-year-old children, both of whom developed diarrhoea the next day. Three untyped virus isolates were obtained from the 24 samples (10 controls and 14 recovered patients) in February, and at the same time 1 isolate of echovirus type 11 and another of Coxsackie B5 were obtained from the 19 patients with diarrhoea. These isolates were obtained in both HT-29 and SKCO-1 cell lines. Echovirus 11 did not grow in HRT-18 monolayers.

Twelve CPE-producing agents (Echo 7, 1; Echo 30, 2; Polio 2, 1; Coxsackie B_6, 2; untyped, 6) were isolated from the 24 stool samples obtained from 13 patients with diarrhoea in July 1983. At the same time, 22 isolates (Echo 7, 5; Echo 30, 2; Polio 2, 7; untyped, 8) were obtained from the 48 stool samples provided by unaffected family members who acted as controls. Analysis of the details of isolations from patients and controls (Table 2) did not reveal any significant differences in virus isolation. In some individuals the same virus was isolated on different days while in others different viruses were found. Members of the same family tended to have the same virus at the same time. Echovirus type 11 was not isolated from any of the 72 stool samples.

Membrane-bound clusters of 20–26 nm small round virus-like particles were seen in the stool of 9 of the 15 individuals from whom echovirus type 11 was cultured in January 1983. None of the other viruses capable of causing diarrhoea, especially rotavirus or Norwalk-like agents, were found in any of the stool samples.

Neutralizing antibody titres of echotype 11 were low in controls and patients during the first few days of diarrhoea in January but increased with increasing duration from onset of diarrhoea (Fig. 2).

Bacterial isolations

Recognized enteric bacterial pathogens were not cultured from the stools of the 18 patients studied in January, while a single isolate of *Salmonella* group C

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was obtained from one of the eight controls. From the 24 stool samples from controls and recovered patients in February two entereopathogenic serotypes of *E. scherichia coli* and one *Shigella flexneri* was isolated, while from the 19 patients with diarrhoea there were two isolations of enteropathogenic serotypes of *E. coli*. From the 13 patients with diarrhoea studied in July, 5 isolations of *Shigella flexneri* were obtained, while 1 *Shigella flexneri* was isolated from the controls. A further 14 patients with diarrhoea were admitted to the metabolic ward in June, July and August and *Shigella flexneri* was cultured from 5.

Clinical studies

The cases occurring from December to March had a median duration of 11 days. In contrast to this, the median duration of the patients affected between May and November was only 7 days. However, in the patients who had a second attack of diarrhoea during May to November the median duration was 16 days. The illness during December to February was characterized by an abrupt onset with watery or semi-formed stools, with a few patients complaining of mucus but no blood in the stool. About a quarter of the patients gave a history of fever associated with the onset of diarrhoea. There was some loss of appetite but vomiting was not a major feature. During the period May–November, the onset of diarrhoea was insidious: the stools were usually small in quantity, the majority gave a history of mucus in the stool while about half had blood. During this whole period (14 months), no cases of encephalitis or meningitis were identified in the village.

A total of 33 patients (19 during January and February and 14 during June, July and August) were admitted to the metabolic ward. Four of the 19 patients admitted during January had transient steatorrhoea that lasted for about 2 weeks from the onset of diarrhoea. Xylose and vitamin B_{12} absorption was normal in all of them. Tests of intestinal absorption were normal in other patients admitted in January and during the May–November period. No specific therapy was given and all recovered. Four deaths occurred in the village due to diarrhoea: three prior to the arrival of the field team in the village and one, a 4-month-old child with intercurrent pneumonia whose parents refused hospitalization, in May 1983.

DISCUSSION

The epidemic of diarrhoea in Punganoor, from December 1982, was characterized by two distinct waves. The first wave of the epidemic, from December 1982 to March 1983, had a sharp peak in January and was associated with a high rate of isolation of echovirus 11 and patients had a longer median duration of illness. A few patients had transient steatorrhoea. The second wave of the epidemic from May to September was spread out. *Shigella flexneri* was isolated from several patients, the median duration of illness was shorter and many patients had blood and mucus in the stool. The higher attack rates and longer median duration during the second wave in patients who had had diarrhoea in the first wave suggests that the agent responsible for the first wave did not provide protective immunity against the agent causing the second wave. In fact, the individuals who were affected in the first wave were more susceptible and recovered more slowly.

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The causal role of a microbial agent found in stool samples from patients with diarrhoea cannot be taken for granted. The absence of other recognized enteric pathogens, a definite serological response indicating an infection and the occurrence of diarrhoea within a short period in controls from whom the agent was isolated would support an aetiological role. In the case of viral agents, if electron microscopic evidence of multiplication in enterocytes and colonocytes can be obtained it would further support the aetiological hypothesis. An ideal way of demonstrating causality would be to challenge susceptible volunteers with the agent and document the occurrence of diarrhoea. The isolation of the echovirus type 11 from 14 of 18 patients with diarrhoea and the serological response (Fig. 2) established that an epidemic of echovirus type 11 infection was present in the village at the same time as the first wave of the epidemic of diarrhoea. No other bacterial or viral agent could be implicated consistently and the occurrence next day of diarrhoea in two asymptomatic control children from whom echovirus was cultured further supports a causal role which could have been confirmed if the growth and multiplication of the virus in gastrointestinal epithelium could have been documented. Transient steatorrhoea has been documented in some patients with echovirus 11-associated diarrhoea (Wenner, 1972).

Echovirus 11 infections, especially in children, have been associated usually with neurological symptoms, particularly meningoencephalitis as well as myalgia, cutaneous eruptions and carditis (Miller *et al.* 1968; Krous, Dietzman & Roy, 1973; Nagington *et al.* 1978; Suzuki *et al.* 1980; Mulcahy, Hanton & de Silva, 1983). Echovirus 11 is also recognized as one of the few echo serotypes which has been associated with diarrhoea, although the causative role of enteroviruses in diarrhoea is still not definite (Wenner, 1972). Some studies (Suzuki *et al.* 1980) suggested that symptomatology with echovirus 11 infection was age-related and that gastrointestinal illnesses occurred with increasing frequency in older children, although a similar trend was not observed by others (Mulcahy, Hanton & de Silva, 1983).

The isolation of a variety of enteroviruses in colonic tumour-derived differentiated epithelial cell lines, especially in July, suggests that these cells might be a good alternative for the field isolation of enteroviruses, for which primate cell strains and cell lines and suckling mouse inoculations are necessary at present (Davis & Phillpotts, 1974; Wenner, 1972). The virus isolations in July confirmed that there is a high rate of enterovirus infection in this population (Feldman *et al.* 1970; Mathan & Baker, 1971) and that the majority of such isolates are obtained usually from people without clinical illness. While some of these episodes of virus shedding are of short duration, the significance of the high prevalence of faecal excretion of enteroviruses in this and other populations from tropical developing countries is not yet fully understood.

Acute diarrhoeal diseases are a major cause of morbidity and mortality in many tropical developing countries. The three deaths in the village prior to the arrival of the field team emphasize the possibility of high mortality due to diarrhoeal epidemics and, from the virtual absence of deaths subsequently, the efficacy of simple measures to maintain hydration orally. The data presented here suggests that epidemics of diarrhoea may be associated with enterovirus infections. The wide prevalence of enterovirus infection in such populations raise important questions regarding the pathogenesis and prevention of such epidemics.

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