Cytotoxic and cell vacuolating activity of *Vibrio fluvialis* isolated from paediatric patients with diarrhoea

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Vibrio fluvialis is a halophilic *Vibrio* species associated with acute diarrhoeal illness in humans. It has the potential to cause outbreaks and has an association with paediatric diarrhoea. In this study, 11 *V. fluvialis* strains isolated from hospitalized patients with acute diarrhoea at the Infectious Diseases Hospital, Kolkata were extensively characterized. All the strains showed growth in peptone broth containing 7 % NaCl. The strains showed variable results in Voges–Proskauer test and to a vibriostatic agent. There was also variation in their antibiograms, and some of the strains were multidrug resistant. Among the 11 strains, two showed only a single band difference in their PFGE profile and the remaining strains showed nine different PFGE patterns. However, unlike PFGE, the strains exhibited close matches and clustering in their ribotype patterns. The haemolytic effect on sheep red blood cells varied with strains. Partial sequence analysis revealed that the *V. fluvialis* haemolysin gene has 81 % homology with that of the El Tor haemolysin of *Vibrio cholerae*. A striking finding was the capability of all the strains to evoke distinct cytotoxic and vacuolation effects on HeLa cells.

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INTRODUCTION

During the late 1970s, a previously unidentified group of vibrios was isolated from diarrhoeic stools of infants, children and young adults in Bangladesh (Huq *et al.*, 1980). These vibrios were designated 'group F' vibrios in England (Furniss *et al.*, 1977) and 'CDC group EF6' in the USA (Huq *et al.*, 1980). Taxonomic studies concluded that these organisms represented a new species, and they were designated as *Vibrio fluvialis* (Lee *et al.*, 1981).

The distribution of *V. fluvialis* is worldwide (McNicol *et al.*, 1980; Thekdi *et al.*, 1982) and this organism is not only isolated from human diarrhoeal cases (Huq *et al.*, 1980; Tacket *et al.*, 1982; Thekdi *et al.*, 1982; Hickman-Brenner *et al.*, 1984; Klontz *et al.*, 1994; Hodge *et al.*, 1995) but also from marine and estuarine environments (Seidler *et al.*, 1980; Lee *et al.*, 1981; Morris & Black, 1985). There are reports of food poisoning caused by this organism (Kobayashi & Ohnaka, 1989; Thekdi *et al.*, 1990), especially due to consumption of raw shellfish (Levine & Griffin, 1993). *V. fluvialis* is also

associated with extraintestinal infections (Yoshii *et al.*, 1987; Albert *et al.*, 1991). The clinical symptoms of the disease include mild to moderate dehydration, vomiting, fever, abdominal pain and diarrhoea (Seidler *et al.*, 1980).

The halophilic *V. fluvialis* (Lee *et al.*, 1981; Lockwood *et al.*, 1982) phenotypically resembles *Aeromonas* species (Seidler *et al.*, 1980) and taxonomically lies between *Aeromonas* and *Vibrio* species (Thekdi *et al.*, 1990). Among the halophilic vibrios, it has close similarity to *V. furnissii*, but, unlike *V. fluvialis*, *V. furnissii* is aerogenic in nature (Brenner *et al.*, 1983).

Since *V. fluvialis* enteritis is reported infrequently, the epidemiology of this infection is not adequately understood. There is very little information available on the virulence factors associated with infection and much less information on the mechanism of pathogenicity of this organism. In this study, we demonstrated that *V. fluvialis* strains are sporadic causes of paediatric diarrhoea and produce factor(s) that evoke cytotoxic and cell vacuolation effects on HeLa cells.

METHODS

Bacterial strains. Eleven *V. fluvialis* strains obtained from the culture collection of NICED, Kolkata, India, were included in this study. These strains were isolated from hospitalized patients admitted either to the Infectious Diseases Hospital or B. C. Roy Children's Hospital, Kolkata, with acute diarrhoea between 1998 and 2000. These patients were part of the routine surveillance sampling of diarrhoeal patients seen at the above hospitals, and relevant clinical information was routinely collected by means of a standard questionnaire. The information requested included age, sex, clinical picture (fever, vomiting and dehydration status), type and duration of diarrhoea, and history of antibiotic therapy prior to the clinic visit.

Stool samples or rectal swabs collected from the diarrhoeal patients were inoculated into alkaline peptone water [1% bacto peptone (Difco), 1% NaCl, pH 8.5] and incubated overnight with shaking at 200 r.p.m. One loopful of enriched sample was plated onto thiosulphate citrate bile salts sucrose agar (TCBS), followed by incubation at 37 °C overnight. Yellow colonies on the TCBS plates were first examined by a single-tube multi-test medium (Kaper, 1979; Nair *et al.*, 1987; Ramamurthy *et al.*, 1993). Growth from this medium was also tested for oxidase reaction by Kovacs method (Cowan, 1979). Oxidase-positive strains that gave a typical alkaline slant acid butt reaction in the multi-test medium and did not agglutinate with *Vibrio cholerae*-specific antisera (O1 and O139) were further biochemically characterized in the API 20E identification system (bioMérieux). Salt tolerance was determined by growth of strains at 37 °C in 1% peptone broth without NaCl or supplemented with 7% NaCl. Susceptibility to 150 µg of vibriostatic compound O/129 (2,4-diamino-6,7-diisopropylpteridine phosphate) was determined in nutrient agar (Ramamurthy *et al.*, 1992). The string test was performed using 0.5% sodium deoxycholate solution with fresh cultures grown on nutrient agar (Kay *et al.*, 1994). The presence of other common enteric pathogens was also examined by standard procedures (WHO, 1987).

Antimicrobial susceptibility testing. Antimicrobial susceptibility of *V. fluvialis* strains was determined by the disc diffusion technique using commercial discs (Hi Media). The following antibiotic discs were used: ampicillin (10 µg), chloramphenicol (30 µg), co-trimoxazole (25 µg), ciprofloxacin (5 µg), furazolidone (100 µg), gentamicin (10 µg), neomycin (30 µg), nalidixic acid (30 µg), norfloxacin (10 µg), streptomycin (10 µg) and tetracycline (30 µg). *Escherichia coli* strain ATCC 25922, which is sensitive to all antibiotics, was used for quality control. Characterization of the strains as susceptible, reduced susceptibility and resistant was based on the size of inhibition zones around each disc, as given in the manufacturer's instructions, which matched the interpretive criteria recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 2002).

PCR. The method for DNA extraction was described in detail elsewhere (Murray & Thomson, 1980). Purified genomic DNA was used as the template for all the PCR experiments. Primer pairs targeting *ctxA* and *tcpA* (classical and El Tor biotypes of *V. cholerae* O1) were used in a multiplex PCR format as described elsewhere (Keasler & Hall, 1993). PCR assays were performed to detect the presence of *V. cholerae* regulatory genes *toxR* (Miller *et al.*, 1987) and *toxT* (Carroll *et al.*, 1997), and other genes like *aldA*, *tagA*, *int* (Kovach *et al.*, 1996; Karaolis *et al.*, 1998; Mitra *et al.*, 2001), *hlyA* (El Tor haemolysin) specific for *V. cholerae* (Manning *et al.*, 1984; Alm *et al.*, 1988; Mitra *et al.*, 2000), *V. fluvialis* haemolysin gene (Kothary *et al.*, 2003) and *stn* (Ogawa *et al.*, 1990). The species-specific *ompW* gene of *V. cholerae* was used in a PCR assay to confirm that the strains identified as *V. fluvialis* were not *V. cholerae*. (Nandi *et al.*, 2000). PCR assays were also performed to check the presence of the regulatory gene *toxR* and virulence genes like *tdh* and *trh* of *Vibrio parahaemolyticus* (Tada *et al.*, 1992; Kim *et al.*, 1999).

All the PCR assays were performed in an automated thermal cycler (Perkin-Elmer). Amplified products were electrophoresed in 2% agarose gel (SRL), stained with ethidium bromide (Sigma), visualized and documented using a Vedio Documentation System (Pharmacia Biotech).

Sequencing of *V. fluvialis* haemolysin gene. The 395 bp PCR product of *V. fluvialis* haemolysin gene was electrophoresed in 1% agarose gel and purified (Qiagen Kit), and both the strands were sequenced in an automated sequencer (ABI PRISM 310 Genetic Analyser, Applied Biosystems). The sequences were aligned using the DNASIS (version V2-1) software programme (Hitachi), and searches for identical sequences were performed using the Basic Local Alignment Search Tool (BLAST) programme available on the National Centre for Biotechnology Information network server.

16S rDNA sequencing. For 16S rDNA sequencing, chromosomal DNA was extracted using Prep Man, Sample Preparation Reagent and prepared according to the manufacturer's protocol (Applied Biosystems). Extracted DNA was used as the template for PCR amplification using Microseq 500, 16S rDNA PCR Module (Applied Biosystems). A 527 bp 16S rDNA fragment was amplified in a reaction volume of 50 µl (25 µl Microseq PCR master mix, 24 µl sterile water and 1 µl chromosomal DNA). The amplified products were purified using a Microcon 100 column (Amicon) as recommended by manufacturer. Forward and reverse sequencing reactions were performed from the amplified product. The sequencing reactions consisted of 13 µl Microseq sequencing mix, 4 µl sterile molecular grade water and 3 µl purified amplified product. The products of the sequencing reactions were purified with Dye Ex Spin Kit (Qiagen), and all sequence analysis was performed in an automated DNA sequencer (Applied Biosystems). Nucleotide sequences generated were aligned and analysed for identification of bacterial species using MicroSeq Analysis software (version 1.40, Applied Biosystems). The database comparison, using the Full Alignment Tool of the MicroSeq software, generated a list of the closest matches with a distance score. This distance score indicated the percentage difference between the unknown sequence and the database sequence. For the purpose of comparing an isolate's original identification to its MicroSeq identification, the Microseq identity was considered to be the closest match in the MicroSeq database no matter what the distance score was.

PFGE. Genomic DNA of *V. fluvialis* strains was prepared in agarose plugs as described previously (Kurazono *et al.*, 1994) and digested with 50 U of *NotI*. PFGE was done by the contour-clamped homogeneous electric field method using a CHEF Mapper system (Bio-Rad) with 1% PFGE grade agarose in 0.5× TBE buffer [44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA (pH 8.0)] for 40 h 24 min. A DNA size standard (λ ladder; Bio-Rad) was used as molecular mass standard. A minichiller (model 1000, Bio-Rad) was used to maintain the temperature of buffer at 14 °C. Run conditions were generated by the auto-algorithm mode of a CHEF Mapper system with a size range of 20–300 kb. Gels were stained in distilled water containing 1.0 µg ethidium bromide ml⁻¹ for 30 min, destained several times and photographed under UV light using the Gel Doc 2000 (Bio-Rad).

Ribotyping. Genomic DNA was digested overnight with *BglI* (Boehringer) and the fragments were electrophoretically separated on a 1% agarose gel using TAE (Tris-acetate EDTA) buffer. For Southern blotting, the gel was treated successively in 0.25 M HCl for 10 min, to allow partial depurination and cleavage of large fragments, in denaturation solution composed of 0.5 M NaOH and 1.5 M NaCl for 30 min and in 0.5 M Tris/HCl (pH 7.4) for 30 min. DNA was then transferred to Hybond-N+ membrane (Amersham) using 20× SSC (3 M NaCl, 0.3 M sodium citrate) by vacuum blotter (Pharmacia). The membrane was washed with 20× SSC and dried at room temperature followed by

fixation in a UV cross-linker (Bio-Rad). A 7.5 kb *Bam*HI fragment of the recombinant plasmid pKK3535 containing an rRNA operon of *E. coli* (Brosius *et al.*, 1981) was used as the *rrn* gene probe for ribotyping. Labelling of the probe, hybridization and detection of bands were carried out according to the instructions of the manufacturer of the ECL detection system (Amersham).

Haemolysin assay. The haemolytic activities of the culture supernates of *V. fluvialis* strains were determined with sheep erythrocytes in a 96-well U-bottom plate (Tarson). The erythrocytes were washed and then diluted to a final concentration of 1 % in sterile 10 mM PBS (pH 7.0). Dilution of culture supernates was made in PBS to measure the haemolysin titre. The mixture was incubated for 1 h at 37 °C and then centrifuged at 2000 r.p.m. for 5 min. The amount of released haemoglobin in the supernate was measured spectrophotometrically at 450 nm.

Tissue culture assay. Brain Heart Infusion broth (BHI, Difco) supplemented with 0.5 % NaCl was used to grow the *V. fluvialis* strains at 37 °C for 18 h in a rotary shaker set at 200 r.p.m. The culture supernate, obtained by centrifugation at 10 000 r.p.m. for 10 min, was filter-sterilized using 0.22 µm filter units (Millipore) and the resultant cell-free culture filtrate was used for tissue culture assay. The cell pellet was then washed, suspended in PBS and sonicated for 2 min on ice using an ultrasonic disintegrator (Tomy). It was then centrifuged at 10 000 r.p.m. for 5 min. After centrifugation and filter-sterilization, the lysate was tested for its effect on tissue cultures.

HeLa and Chinese hamster ovary (CHO) cells were grown as monolayers in tissue culture flasks (25 cm²) using Eagle's Minimum Essential Medium (MEM; Gibco) supplemented with 10 % fetal bovine serum at 37 °C in a humidified 5 % CO₂ atmosphere (Haraeus Instruments). Confluent monolayers of HeLa cells (Fig. 2a) and CHO cells grown for 3–4 days were transferred from the flasks to 96-well plates (about 4 × 10⁵ cells) after trypsinization and maintained in 5 % foetal bovine serum as described above. The culture supernate and the cell lysate of the test strains were serially diluted with sterile Hanks's balanced salts solution (HBSS, Gibco) and aliquots of each test dilution were added in duplicate to the assay plate and incubated for 24 h. Morphological changes and cytotoxic effects were observed after 24 h using an inverted microscope (Olympus). The toxin titre was expressed as the highest dilution that affected 50 % of the cells in a well. BHI, HBSS and PBS served as negative controls in this assay.

Suckling mice assay. The biological activity of the culture supernate was assayed in the suckling mouse model. BHI was the recommended medium for production of toxin(s) causing intestinal fluid accumulation in suckling mice (Nishibuchi & Seidler, 1983). BHI broth supplemented with 0.5 % NaCl was inoculated with the *V. fluvialis* strain and incubated at 37 °C for 18 h with shaking at 200 r.p.m. Cell-free culture supernate prepared by centrifugation (10 000 r.p.m. for 10 min) of the broth culture and by filtration of the supernate through a 0.22 µm membrane filter was tested in suckling mice. Three-day-old Swiss albino suckling mice were used in this study. Prior to the experiment, the suckling mice were separated from their mothers for about 2 h. Three mice were used for each test. A 0.1 ml portion of the culture supernate mixed with Evans blue dye (0.01 %) was administered orally into the stomach of each test animal using a blunt needle and a 1 ml hypodermic syringe. Doses of 0.1 ml PBS (10 mM, pH 7.2) containing 5 ng purified heat-stable toxin of *Vibrio cholerae* non-O1, non-O139 (NAG-ST) and Evans blue were introduced as a positive control and 0.1 ml BHI containing only the dye were introduced as a negative control. All the mice were kept at room temperature for 4 h and then sacrificed using chloroform. The intestine and the stomach were removed and the ratio of the pooled intestine-stomach weight to the remaining body weight was measured to calculate the fluid accumula-

tion ratio by a modification of the method of Baselski *et al.* (1977). A fluid accumulation ratio of ≥0.09 was considered as a positive response.

Rabbit ileal loop assay. An outbred New Zealand white rabbit weighing between 1.5 and 2.5 kg was selected for the rabbit ileal loop assay. The animal was fasted overnight prior to the experiment. Surgery was done under anaesthesia [35 mg ketamine (kg body wt)⁻¹ and 5 mg xylazine (kg body weight)⁻¹; Sigma] given intravenously. The ileal loop procedure was that described by De & Chatterjee (1953). Briefly, the intestine was brought out through a mid-line incision in the abdomen. A suitable length of rabbit ileum starting about 6–8 cm away from the ileocaecal junction was selected for the test. The portion of the small intestine was washed carefully with warm (37 °C) sterile PBS (0.1 M, pH 7.4). A total of five loops were prepared. The lengths of the loop and inter loop spaces were 6 cm and 2 cm, respectively. One millilitre of live bacterial cell suspension of *V. fluvialis* strain in sterile PBS containing about 10⁸ c.f.u. was then introduced into each of three test loops. *V. cholerae* O1 Inaba strain C67O9 was used as a positive control and sterile PBS was used as a negative control. After inoculation, the small intestine of the animal was carefully pushed back inside the abdomen and then the incision was sutured. The animal was kept in its cage and supplied with water. The animal was sacrificed after 18 h and fluid accumulation was measured as the ratio of individual loop fluid volume to loop length, expressed as ml cm⁻¹. A test preparation was considered positive if the ratio was ≥0.9.

RESULTS

With the exception of one strain, all strains of *V. fluvialis* were isolated from diarrhoeal patients whose ages ranged between 1 and 11 years. *V. fluvialis* isolates from five of the 11 patients were associated with mixed infection either with *V. cholerae* or with *V. parahaemolyticus* (Table 1). The clinical manifestation of patients from whom *V. fluvialis* was isolated as sole pathogen (54.5 %) resembled that of cholera. A majority of the patients who were solely infected with *V. fluvialis* had moderate dehydration.

V. fluvialis strains exhibit biochemical traits typical for the genus *Vibrio* with a few features resembling *Aeromonas*. In many cases, the API 20E system identified *V. fluvialis* strains as *A. hydrophila* with high identification scores (Seidler *et al.*, 1980). Therefore, we included a salt-tolerance test to distinguish *V. fluvialis* from the *Aeromonas* species. All the *V. fluvialis* strains grew well in broth containing 1 % peptone and 7 % NaCl. *V. fluvialis* strains that were inoculated in the multi-test medium showed alkaline slant acid butt reaction without production of gas, resembling *V. cholerae*. All the strains were arginine dihydrolase-positive and lysine decarboxylase- and ornithine decarboxylase-negative. Strains CRC111, CRC159 and CRC233 showed positive results in the Voges–Proskauer test. All the strains were positive in the oxidase and string tests. Three strains (PL45, PL78/7b and AN48) were resistant to 150 µg of vibriostatic compound. The results of *V. fluvialis* strains in a variety of biochemical tests included in this study are shown in Table 2. The identification of the *V. fluvialis* strains was confirmed by 16S rDNA sequencing. Antibiotic-resistance patterns of *V. fluvialis* strains are shown in Table 3. Notably, three strains that were resistant to vibriostatic agent, PL45, PL78/7b and AN48, were also resistant to co-trimoxazole (Table 3).

Table 1. Clinical manifestation of acute diarrhoeal patients from whom *V. fluvialis* strains were isolated

+ Positive, – negative, M, male; F, female.

Strain no.	Year of isolation	Patient		Clinical manifestation			Other pathogen
		Age (years)	Sex	Vomiting	Fever*	Dehydration†	
PG39	1998	3	F	–	–	No	None
PG41	1998	4	M	–	–	No	None
PL45	1998	1	M	–	–	Severe	None
PG152	1998	35	F	–	–	Moderate	<i>V. parahaemolyticus</i>
PL78/7b	1998	7	F	+	–	Severe	<i>V. cholerae</i> O1
PL169b	1998	3	M	+	–	Severe	<i>V. cholerae</i> O1
PL171b	1998	2	F	+	–	Severe	<i>V. cholerae</i> O1
CRC111	2000	11	M	+	–	Moderate	None
CRC159	2000	5	M	–	+	Moderate	None
CRC233	2000	2	M	+	–	Moderate	None
AN48	2000	3	F	+	–	Severe	<i>V. cholerae</i> O1

*Defined as ≥ 37.7 °C.

†As defined by the WHO (1995).

PCR assay and sequencing

Since the clinical manifestation of *V. fluvialis* infection resembled cholera and some of the patients were coinfecting either with *Vibrio cholerae* O1 or *Vibrio parahaemolyticus*, all the strains included in the study were screened for the presence of virulence and regulatory genes of *V. cholerae* as well as *V. parahaemolyticus*. All the *V. fluvialis* strains were negative for *V. cholerae* *ctxA*, *tcpA* (Classical/El Tor), *toxR*, *toxT*, *aldA*, *tagA*, *hlyA* (El Tor haemolysin), *stn* and *ompW*, and *V. parahaemolyticus* *toxR*, *tdh* and *trh*. Only CRC111 and CRC233 were positive for the *int* gene, which constitutes a part of the cluster of genes in the VPI (Vibrio Pathogenicity Island) of *V. cholerae*. All the *V. fluvialis* strains produced a 395 bp amplicon of haemolysin gene and sequence analysis revealed that the *V. fluvialis* haemolysin gene had 81% homology with that of El Tor haemolysin of *V. cholerae* in the regions of the gene that were matched (data not shown).

PFGE and ribotyping

We used molecular typing by PFGE and ribotyping to determine whether the *V. fluvialis* strains were clonal. Of the 11 strains, only two (PG39 and PG41) were found to be closely related as assessed by PFGE profiles, while the remaining nine showed distinct PFGE profiles (data not shown). However, in ribotyping a dominant pattern was exhibited by five strains (PG39, PG41, PL78/7b, PL171b and CRC111) (Fig. 1). Among the six remaining strains, three strains, PG152, CRC233 and PL45 (data not shown), were identical in their ribotype pattern and the pattern of AN48 was closely related to them. Another two different patterns were shown by the strains CRC159 and PL169b (Fig. 1). From ribotyping analysis it was found that the strains isolated in 1998 (PG39, PG41, PL78/7b and PL171b) were clonal and

also clonally related to a strain isolated in 2000 (CRC111). Clonality was also observed among PL45, PG152 and CRC233 isolated during 1998, 1998 and 2000, respectively.

Haemolytic effect and tissue culture assay

All the *V. fluvialis* strains were haemolytic for sheep erythrocytes. Except for one strain, PL169b, the haemolysin titre was generally low (Table 4). The cell-free culture filtrates of all the *V. fluvialis* strains were capable of causing cytotoxic (Fig. 2b) and vacuolation (Fig. 2c) effects on HeLa cells. The strains showed the cytotoxic effect with end-point titres ranging from 2 to 64 (Table 4). Among the 11 strains, only two (PL78/7b and PL169b) showed high titres of 64. In addition to cytotoxicity, all the strains showed a cell vacuolating effect but only at the point where the cytotoxic effect on HeLa cells was 50% or less. The effect of the culture supernates and the cell lysate of the *V. fluvialis* strains was also detected with CHO cells. The culture supernates of all the strains were cytotoxic against CHO cells and the highest titre detected was 512. However, when the CHO cells were treated with cell lysate there was a very weak response (Table 5).

Suckling mice and rabbit ileal loop assays

None of the strains induced fluid accumulation in suckling mice. However, purified NAG-ST exhibited distinct fluid accumulation with a fluid accumulation ratio of ≥ 0.09 . To determine the *in vivo* toxigenic response, *V. fluvialis* strain PL169b, which evoked a cytotoxic effect with high toxin titre in the tissue culture assay, was examined in the ligated rabbit ileal loop assay. However, no fluid accumulation was observed, as in the loop containing only PBS. The positive control loop receiving *V. cholerae* O1 C67O9 elicited a high fluid accumulation response, with a fluid accumulation ratio of 1.083.

Table 2. Response of *V. fluvialis* strains in biochemical tests used in this study

+, Positive; –, negative; R, resistant; S, susceptible.

Biochemical test	Test result										
	PG39	PG41	PL45	PG152	PL78/7b	PL169b	PL171b	CRC111	CRC159	CRC233	AN48
ONPG	+	+	+	+	–	+	+	+	+	+	–
Arginine dihydrolase	+	+	+	+	+	+	+	+	+	+	+
Lysine decarboxylase	–	–	–	–	–	–	–	–	–	–	–
Ornithine decarboxylase	–	–	–	–	–	–	–	–	–	–	–
Citrate utilization	–	–	–	–	–	–	–	–	–	–	–
H ₂ S production	–	–	–	–	–	–	–	–	–	–	–
Urease	–	–	–	–	–	–	–	–	–	–	–
Gelatinase	+	+	+	+	–	+	–	+	+	+	+
Indole	+	+	+	+	–	+	–	+	+	+	+
Voges–Proskauer test	–	–	–	–	–	–	–	+	+	+	–
Fermentation of:											
Glucose	+	+	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+
Inositol	–	–	–	–	–	–	–	–	–	–	–
Sorbitol	+	+	–	+	–	–	–	+	+	–	+
Rhamnose	–	–	–	–	–	–	–	–	–	–	–
Sucrose	+	+	+	+	+	+	+	+	+	+	+
Melibiose	–	–	–	–	–	–	–	–	–	–	–
Arabinose	+	+	+	+	+	+	+	+	+	+	+
Oxidase test	+	+	+	+	+	+	+	+	+	+	+
String test	+	+	+	+	+	+	+	+	+	+	+
Growth in absence of NaCl	–	–	–	–	–	–	–	–	–	–	–
Growth in 7 % NaCl	+	+	+	+	+	+	+	+	+	+	+
Susceptibility to O/129 (150 µg)	S	S	R	S	R	S	S	S	S	S	R
Tryptophane deaminase	–	–	–	–	–	–	–	–	–	–	–
Amygdalin	–	–	–	–	–	–	–	+	–	–	–
Growth in TCBS	+	+	+	+	+	+	+	+	+	+	+

Table 3. Antimicrobial-resistance pattern of *V. fluvialis* strains

Abbreviations: A, ampicillin; C, chloramphenicol; Cf, ciprofloxacin; Co, co-trimoxazole; Fz, furazolidone; G, gentamicin; N, neomycin; Na, nalidixic acid; Nx, norfloxacin; S, streptomycin; T, tetracycline.

Strain no.	Antibiogram
PG39	Fz
PG41	Fz
PL45	ACoFzS
PG152	Fz
PL78/7b	ACoNaS
PL169b	Fz
PL171b	ACfFzNaST
CRC111	Fz
CRC159	Fz
CRC233	AFz
AN48	ACCoCfFzNaST

DISCUSSION

The distribution of *V. fluvialis* in the environment and its significance as an aetiological agent of diarrhoea are inadequately understood because of the organism's marked biochemical similarity to *Aeromonas* species. Due to complexity in the identification of *V. fluvialis*, information regarding its incidence among diarrhoeal patients is scanty. In this study, most of the *V. fluvialis* strains were isolated from children with diarrhoea, indicating that children may be the most vulnerable group to this pathogen. Retrospective studies conducted by the British Public Health Laboratories

Table 4. Effect of culture supernate of *V. fluvialis* strains on HeLa cells and haemolytic activity

Strain no.	Haemolytic effect (titre)	Effect on HeLa cells* (titre)	
		Cytotoxic effect†	Vacuolation effect
PG39	2	4	8
PG41	4	32	32
PL45	8	8	8
PG152	4	2	4
PL78/7b	4	64	128
PL169b	32	64	64
PL171b	N‡	2	2
CRC111	N‡	16	32
CRC159	4	8	16
CRC233	2	16	16
AN48	2	16	16

*Strains were grown in 2 ml BHI medium supplemented with 0.5% NaCl. The cell-free culture supernate was added to non-confluent HeLa cells.

†The titre was expressed as the highest dilution of the culture supernate showing cytotoxic effects on 50% HeLa cells in a well.

‡Neat toxin.

Fig. 1. *BglI* ribotyping patterns obtained with *V. fluvialis* strains. Lane 1, PG39; lane 2, PG41; lane 3, PG152; lane 4, PL78/7b; lane 5, PL169b; lane 6, PL171b; lane 7, CRC111; lane 8, CRC159; lane 9, CRC233; lane 10, AN48. Sizes of λ HindIII molecular markers are indicated.

on lysine decarboxylase-negative *Aeromonas* strains showed that one-third of the strains were actually *V. fluvialis* (Lee *et al.*, 1981). Thus, it is essential to include the salt tolerance test to differentiate these two species (Seidler *et al.*, 1980; Lee *et al.*, 1981), although there is a report on *V. fluvialis* strains capable of growing in absence of NaCl (Suthienkul *et al.*, 1985).

All the *V. fluvialis* strains included in this study grew well in peptone water containing 7% NaCl. Three strains were positive in the Voges–Proskauer test. Without the NaCl tolerance test, we would have identified the *V. fluvialis* strains as *A. hydrophila* based on the API 20E Voges–Proskauer test. The strains were not only variable in their antibiogram but some of them were also multidrug resistant. Three of the *V. fluvialis* strains were resistant to vibriostatic agent O/129 (150 µg) and these strains were also resistant to co-trimoxazole. As reported for *V. cholerae* (Ramamurthy *et al.*, 1992), there may be a relationship between O/129 resistance and resistance to co-trimoxazole in *V. fluvialis*.

Recently, 16S rDNA sequencing has become a very useful technique for the accurate identification of micro-organisms (Patel *et al.*, 2000). Using this molecular tool, we confirmed all the strains to be *V. fluvialis*. Molecular typing by PFGE showed less similarity among *V. fluvialis* strains analysed in this study. Only two strains, PG39 and PG41, seemed clonally related as both PFGE and ribotyping exhibited similar results. Amongst the remaining strains, those that were identical in their ribotype pattern showed different PFGE patterns.

V. fluvialis is known to produce several toxins including CHO cell elongation factor (Lockwood *et al.*, 1982), CHO cell killing factor (Lockwood *et al.*, 1982; Wall *et al.*, 1984) and an enterotoxin capable of causing fluid accumulation in

Table 5. Response of CHO cells to culture supernate and cell lysate of *V. fluvialis* strains

Strain no.	Cytotoxic effect (titre*)	
	Culture supernate	Cell lysate
PG39	32	N (< 50 %)
PG41	64	N
PL45	64	N
PG152	64	2
PL78/7b	64	2
PL169b	512	4
PL171b	8	N (< 50 %)
CRC111	16	16
CRC159	128	4
CRC233	64	N
AN48	128	No effect

*The titre value is expressed as the highest dilution of the culture supernate and cell lysate showing cytotoxic effects on 50 % CHO cells in a well. N, neat toxin.

the rabbit ileal loop model (Sanyal *et al.*, 1980; Seidler *et al.*, 1980; Huq *et al.*, 1985; Suthienkul *et al.*, 1985). The organism also produces an enterotoxin similar to that described for non-O1 *V. cholerae* inducing fluid accumulation in suckling mice (Nishibuchi & Seidler, 1983). To our knowledge, this is the first report of *V. fluvialis* showing cytotoxic and vacuolating activity on HeLa cells. These strains are also capable of causing haemolysis of sheep red blood cells.

Only a few other bacterial toxins with vacuolating activity have been identified so far. *Helicobacter pylori* causes vacuolation of eukaryotic cell lines. About 50 % of *H. pylori* isolates have a proteinaceous cytotoxin, VacA, that induces cytoplasmic vacuolation in eukaryotic cells (Leunk *et al.*, 1988; Cover & Blaser, 1992). A recent report has demonstrated a new role for El Tor haemolysin of *V. cholerae*: that it induces vacuolation in mammalian cells (Mitra *et al.*, 2000). In *V. cholerae*, this vacuolating phenomenon was masked by the effects of cholera toxin in toxigenic strains and cell-rounding factor in non-toxigenic strains (Mitra *et al.*, 2000), which may be the reason why this manifestation was not reported earlier. In this study, we showed that the crude toxin (culture supernate) has both cytotoxic and vacuolating effects on HeLa cells. Our study also supports the fact that the nucleotide sequence of the *V. fluvialis* haemolysin gene shares significant homology with that of the El Tor haemolysin of *V. cholerae* (Kothary *et al.*, 2003). However, it is still not clear whether the El Tor-like haemolysin of *V. fluvialis* has any correlation with vacuolation effects on HeLa cells similar to *V. cholerae*.

Fig. 2. Photomicrograph of HeLa cells with (a) confluent growth and effect after incubating the cells at 37 °C for 24 h with culture supernate showing (b) cytotoxic effect and (c) vacuolation effect.

As all the *V. fluvialis* strains have shown both cytotoxic and cell vacuolating activity on HeLa cells, it is difficult to determine whether a single toxin or multiple toxins are responsible for this dual effect. In this background, we assume that there may be two possibilities: either (i) a distinct toxin is responsible for both the effects, exhibiting a cytotoxic effect in higher concentration and a vacuolating effect when diluted, or (ii) two independent toxins are responsible for the cytotoxic and cell vacuolation effects but the toxin responsible for the cytotoxic effect masks the vacuolating effect at higher concentrations. In addition, the variable titre of cytotoxicity in the HeLa cell assay indicates that there may be variation in gene expression among strains. The effect of culture supernate and cell lysate of all the *V. fluvialis* strains was also tested with CHO cells. The cytotoxic effect that was detected in the CHO cells treated with the culture supernate was possibly due to the presence of *V. fluvialis* haemolysin (Kothary *et al.*, 2003). None of our strains was found to contain the cell elongation factor in their cell lysate as reported earlier (Lockwood *et al.*, 1982).

The responses of the suckling mice to orally administered culture supernates were measured by intestinal fluid accumulation. Since the culture supernate of *V. fluvialis* strains failed to evoke fluid accumulation in the suckling mice assay, it can be assumed that none of the strains produced heat-stable toxin. Interestingly, the *V. fluvialis* haemolysin, a heat-labile toxin, can induce fluid accumulation in suckling mice in a dose-dependent manner (Kothary *et al.*, 2003). For this reason, with the tested strains we consider that the amount of haemolysin present in the culture supernate was not sufficient to elicit fluid accumulation. The rabbit ileal loop assay also did not respond to the toxigenic strain PL169b.

Enteric pathogens secrete pore-forming toxins, which are important determinants of virulence in humans and animals. Similar to *V. cholerae* haemolysin, aerolysin is a pore-forming toxin secreted by the human pathogen *A. hydrophila* that causes vacuolation in the cytoplasm of BHK (baby hamster kidney) cells (Abrami *et al.*, 1988). A phenotype similar to that of aerolysin-induced vacuolation has also been observed with *Serratia marcescens* haemolysin (Sh1A) (Hertle *et al.*, 1999). Considering the above-mentioned findings, we assume that the vacuolating toxin may play a role in the pathogenesis of *V. fluvialis*. The interesting feature is that, like *V. cholerae* non-O1 non-O139, *V. fluvialis* also produces a variety of toxins and a single strain can produce different types of toxins (Lockwood *et al.*, 1982). However, the involvement of vacuolating cytotoxin in *V. fluvialis*-mediated disease is yet to be demonstrated. A detailed investigation is needed to evaluate the role of the toxin(s) in the pathogenesis of the enteric disease and to determine its mechanism of action on target cells. The relationship of the *V. fluvialis* vacuolating cytotoxin to other known vacuolating toxins of different bacteria also needs to be explored further.

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