Epidemiol. Infect. (1998), **121**, 67–76. Printed in the United Kingdom © 1998 Cambridge University Press

The prevalence of *Vibrio* spp. in drinking water and environmental samples in Vellore South India

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(*Accepted 13 March 1998*)

SUMMARY

The prevalence of *Vibrio cholerae* in drinking water, lakes and sewage outfalls during July and August 1996 in Vellore, India was determined. Drinking water samples were collected on single occasions from 12 sites in different geographic areas of the town where cholera had been reported. Samples of water, plankton and sediment were collected from fixed sites at three lakes on three occasions separated by at least 3 days during the course of the study. Samples from open sewers were taken from two representative sites in four areas of the town. Bacteria isolated from samples were identified by standard biochemical tests and isolated strains of *V. cholerae* tested for their ability to agglutinate O1 and O139 antisera. Water samples from lakes were also tested for the presence of *V. cholerae* O1 and O139 by fluorescent antibody staining. Non-O1, non-O139 strains of *V. cholerae* were detected in 41% of drinking water samples and 100% of water, sediment and plankton samples from the test lakes. Eighty-seven per cent of open sewers sampled contained viable non-O1, non-O139 *V. cholerae*. Fluorescent antibody staining gave positive results for *V. cholerae* O1 and O139 for all water samples from the three lake sites. Strains of *Aeromonas* spp. were isolated from 58% of drinking water samples and from 66% of sediment, 77% of plankton and 55% of water samples from lakes. All open sewers sampled contained *Aeromonas* spp. PCR amplification employing specific primers demonstrated that none of the non-agglutinating *V. cholerae* isolates contained the *ctx* operon. The non-O1, non-O139 *V. cholerae* isolates showed different patterns of antibiotic resistance to ampicillin, ciprofloxacin, chloramphenicol, tetracycline and trimethoprim.

INTRODUCTION

Cholera is an epidemic disease of major global and public health significance. The causative organism *Vibrio cholerae* can be divided into two major groups O1 and non-O1 on the basis of the somatic O antigen. Although epidemics of O1 cholera occur regularly, the non-O1 serogroup has traditionally been associated only with sporadic cases. However, in 1992, a new non-O1 serotype, O139, appeared causing outbreaks of cholera in India and Bangladesh [1]. Since the initial appearance of *V. cholerae* O139 studies have documented the appearance and disappearance of serotypes O1 and O139 and detailed molecular analysis of strains has shown that there is continual evolution of new O1 and O139 strains [2, 3].

At the department of Clinical Microbiology, Christian Medical College Hospital, Vellore the clinical isolation rates of *V. cholerae* O1 and O139 has been carefully monitored; demonstrating that there has been a fluctuation in the relative prevalence of the

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https://doi.org/10.1017/S0950268898001010 Published online by Cambridge University Press

two serotypes between 1992 and 1996 [4, 5]. During 1996, clinical cases of cholera-like illness occurred each month in Vellore, the majority of isolates from these patients were *V. cholerae* O1 although non-O1, non-O139 *V. cholerae* and *Aeromonas* spp. have also been isolated. There have been very few isolates of *V. cholerae* O139 in contrast to previous years [5]. The reasons for these fluctuations in the clinical pattern of cholera in Vellore are not clear but may result from environmental factors. Natural water bodies have been implicated as sources and reservoirs of *V. cholerae*; however, the precise role of the natural environment in the transmission and epidemiology of cholera is not clear. Although the recent suggestion that *V. cholerae* can survive in a viable but non-culturable state in the environment and the association of *V. cholerae* with plankton may shed new light on the behaviour of this organism in the natural environment [6, 7, 8].

An understanding of the aquatic environment probably holds the key to understanding the epidemiology of cholera and, in particular, the events surrounding the emergence of new toxigenic clones of *V. cholerae*. In addition, local factors may greatly influence the transmission of cholera in a given area and a thorough understanding of these is required before effective control strategies can be proposed. Although the clinical incidence of cholera in Vellore has been well studied nothing is known of the prevalence of *V. cholerae* and other diarrhogenic aquatic organisms in the environment. The aim of this study was to determine the prevalence of different *V. cholerae* strains in environmental and drinking water collected in the Vellore area in order to gain a more comprehensive knowledge of the occurrence and niche of *V. cholerae* serotypes in the environment in Vellore to provide a baseline for future studies and in addition to detect possible vehicles of transmission.

METHODS

Source of samples

Samples were collected in and around the town of Vellore which is located 120 km west of Madras, India in July and August 1996. Samples of surface water, soil sediment (mud from the boundaries) and plankton were collected from three water bodies in and around Vellore (Fig. 1). Two of the sample sites, Oteri and Ondra Thankal were fresh water pools located on the outskirts of Vellore town to the North and South. Both pools were used for washing clothes and utensils, bathing and as a source of drinking water for domesticated animals. The third site was fresh water moat located in the heart of Vellore town. The moat is heavily used for bathing and washing and in addition is a source of fish which is caught and sold in the local market. Water samples were collected from drinking water taps from specific areas of the town where cholera cases had previously been identified and from the pumping station immediately after treatment and chlorination (Fig. 1). Sewage samples were taken from open sewers located within 1 m of water standpipes.

Collection of samples

Drinking water taps. Water was allowed to flow for 2 min from standpipes and collected in sterile flasks.

Water bodies. Water was collected 2–3 m away from the bank and at a depth of 30 cm in sterile flasks. Flasks were held at the bottom and the mouth immersed 30 cm deep into the water. Soil sediment samples were collected from the boundaries of the water bodies at a depth of 15 cm using a core sampler and were transferred immediately to a sterile container. Plankton was collected 5–10 m away from the boundary of water bodies by towing a 10 cm × 10 cm plankton net across the water attached to a fishing line. The net was transferred to a sterile Ziploc plastic bag and then transported to the laboratory.

Sewage. Samples were collected with cotton gauze swabs from open sewers and transported to the laboratory in a sterile test tube.

Sample processing

All the water samples were filtered through an HA membrane filter of 0·45 μm pore size (Millipore, Bedford, USA). The membrane was then inoculated into 20 ml of alkaline peptone water (APW). Soil sediments were inoculated into an equal volume of double strength APW. The Plankton net was placed into 20 ml of alkaline peptone water. Sewage swabs were taken in duplicate, one was enriched in 5 ml of APW while the other one was streaked directly onto blood agar (BA), MacConkey agar (MA) and Thiosulphate Citrate Bile Salt Sucrose Agar (TCBS) solid media. All the APWs were incubated at 37 °C for 6 h and subsequently subcultured onto the appropriate solid media as above.

Identification of isolates

After overnight incubation, β-haemolytic colonies on

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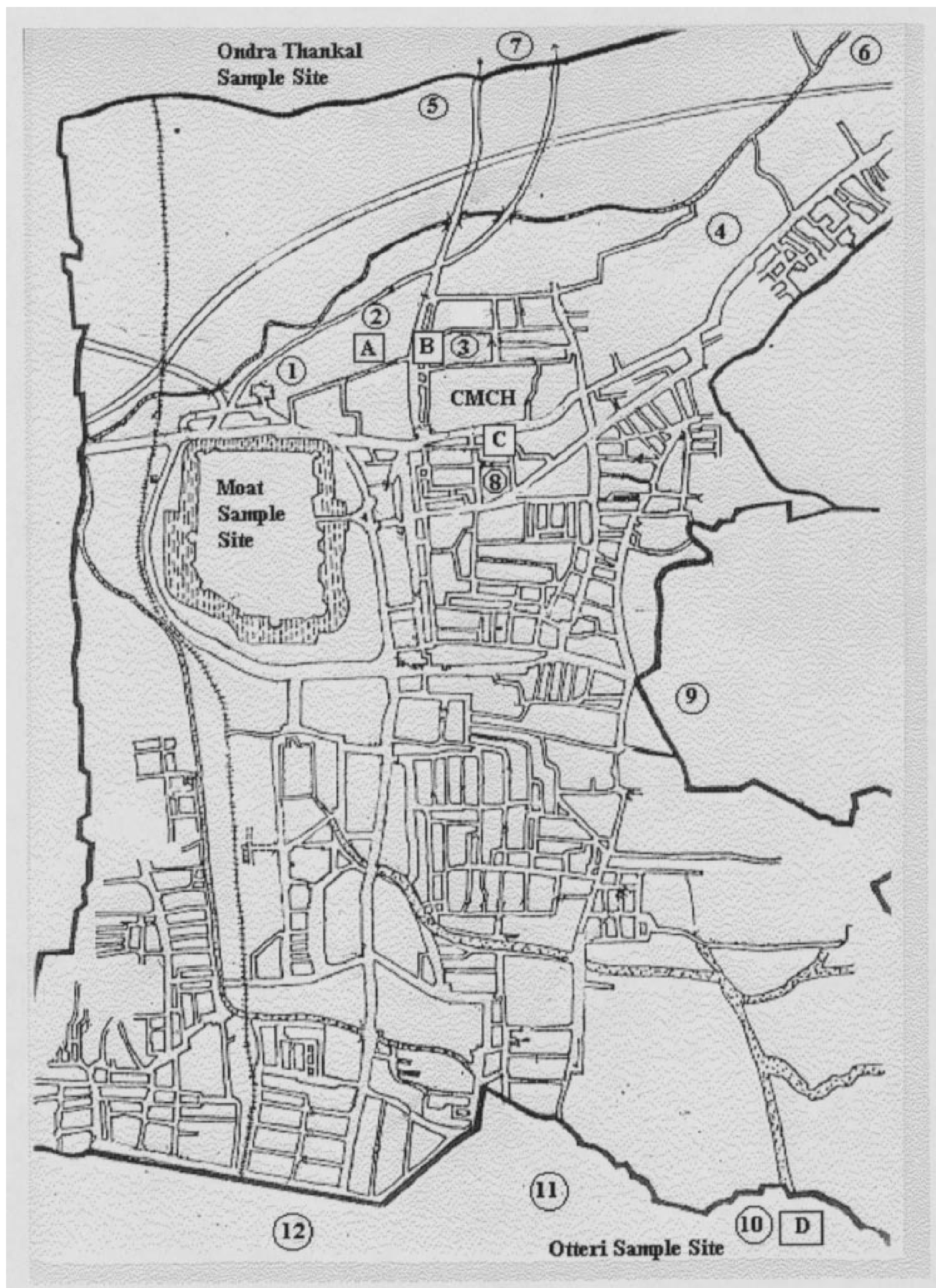

Fig. 1. Vellore Town showing sample sites. ○, drinking Water sample site; □, drainage site. The three surface water sites are shown by name.

BA, flat-non lactose fermenting colonies on MA and yellow colonies from TCBS were subjected to the oxidase test. Oxidase positive colonies were further characterized employing standard procedures. Isolates were inoculated into primary screening media Mannitol motility (MM), triple sugar iron agar (TSI) and peptone water for indol (PW). They were also tested for production of lysine, ornithine decarboxylase and arginine dihydrolase. *Vibrio cholerae* was confirmed if strains were positive for MM, TSI and

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Table 1. *Speciation of* Aeromonas *isolates*

	A. caviae	*A. hydrophila*	*A. sobria*
Gas from glucose	−	+	+
Salicin	+	+	−
Esculin	+	+	−
VP	−	+	+
6·5% NaCl	−	−	−

PW, lysine and ornithine were decarboxylated but arginine dihydrolase was negative. After confirmation by biochemical tests, all *V. cholerae* were tested with polyvalent O1 and O139 antisera in slide agglutination tests. Other organisms isolated were identified by standard biochemical tests. *Aeromonas* species were further speciated based on biochemical reactions Table 1.

Detection of *Vibrio cholerae* O1 and O139 by fluorescent antibody

Samples from water bodies were screened for the presence of *Vibrio cholerae* O1 and O139 by a modification of the method described by Xu and colleagues [9]. Ten ml of water from each site was centrifuged and the resultant pellet resuspended in 100 μl sterile distilled water. Twenty μl samples were placed on slides, air dried and fixed with acetone for 10 min. Ten μl of test antiserum (either polyvalent O1 or O139 supplied from the National Institute of Cholera and Enteric Diseases, Calcutta) was added to each well and the slides incubated in a moist chamber at room temperature for 30 min. Slides were then washed in PBS pH 7·6 twice. Twenty μl of fluorescein labelled conjugate (Sigma, Dorset, UK) was then added to each well and the slides incubated and washed as above. Slides were mounted with buffered glycerol saline and examined by fluorescent microscopy employing a Leitz Ortholux II microscope-Filter I 2/3, blue, wavelength 450–490 nm. Each sample was set up in duplicate and tested with O1 and O139 antibody. The specificity of the test was confirmed by testing positive controls *Vibrio cholerae* O1 and O139 and as negative controls standard strains of *Escherichia coli*, *Pseudomonas aeruginosa*, non-O1 non-O139 *V. cholerae* and representative strains of *Aeromonas* spp. and non-agglutinating *V. cholerae* which had been isolated from the test samples during the course of the survey. Control organisms were inoculated into 10 ml of sterile distilled water and examined as for the test samples.

Detection of the *ctx* operon by PCR

The non-agglutinating isolates obtained were screened for the presence of the *ctx* operon by PCR employing the following primers [10]:

5′-CTCAGACGGGATTTGTTAGGCACG-3′
3′-GCATTATCCCCGATGTCTCTATCT-5′

Two colonies of each test strain were picked and emulsified in 100 μl of sterile distilled water and boiled for 90 sec. Five μl was used as the DNA template. The PCR reaction conditions were as described by Shirai and colleagues [10]. Samples were subjected to a cycle of amplification steps consisting of 1 min at 94 °C, 1·5 min at 60 °C and 1·5 min at 72 °C. This round of amplification was repeated for a further 39 cycles. Standard isolates of toxigenic *V. cholerae* (O1 and O139) and non-toxigenic *V. cholerae* (non-O1 and non-O139) were employed as positive and negative controls respectively.

Antimicrobial sensitivity testing

The minimum inhibitory concentrations of amoxycillin (SmithKline Beecham Pharmaceuticals, Surrey, UK), ciprofloxacin (Bayer, Newbury, UK), chloramphenicol (Boehringer–Mannheim, UK), tetracycline (Lederle Laboratories, Gosport, UK) and trimethoprim (GlaxoWellcome, Crewe, UK) were determined for the non-O1 non-O139 *V. cholerae* environmental isolates. The procedures followed were as described in the guidelines of the British Society for Antimicrobial Chemotherapy [11]. The control strains employed were *Pseudomonas aeruginosa* NCTC 10662, *Staphylococcus aeureus* NCTC 6571 and *Escherichia coli* NCTC 10418.

RESULTS

Drinking water

Culturable organisms isolated from the 12 drinking water samples taken are shown in Table 2. Five

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Table 2. *Organisms isolated from drinking water taps*

Location	Date	NAG*	*Aeromonas* sp.	Others
1	24/7/96	–	+	NFGNB†
2	26/7/96	+	+	–
3	29/7/96	–	–	–
4	15/7/96	–	+	ASF,‡ NFGNB
5	3/7/96	+	–	NFGNB, *Proteus mirabilis*, *E. coli*, *Plesiomonas* spp., *Enterobacter* spp.
6	12/7/96	–	–	ASF, NFGNB
7	11/7/96	+	–	NFGNB, *Enterobacter* spp.
8	30/7/96	+	+	–
9	5/7/96	–	–	–
10	29/7/96	–	+	NFGNB
11	9/7/96	+	+	NFGNB, *E. coli*, *Plesiomonas* spp., *Enterobacter* spp.
12	10/7/96	–	+	NFGNB, *Plesiomonas* spp.

* NAG, non-O1, non-O139 *V. cholerae*.
† NFGNB, non fermenting Gram negative Bacillus.
‡ ASF, aerobic spore former.

Table 3. Aeromonas *spp. isolated from drinking water and environmental samples*

	Number of isolates	% Isolates	
		Drinking water	Environment
A. hydrophila	30	23·3	76·6
A. sobria	29	13·7	86·3
A. caviae	1	0	100

samples yielded culturable non-O1, non-O139 *V. cholerae* and three of these also contained a variety of other organisms including members of the Enterobacteriaceae. Two samples contained no detectable viable bacteria, interestingly one of these (sample 9) was from the treatment plant from which water is pumped to areas of the town. Seven of the samples were positive for viable *Aeromonas* spp. and both *Aeromonas hydrophila* and *Aeromonas sobria* were identified (Table 3). Three specimens grew *Plesiomonas shigelloides* in addition to the above.

Environmental samples

Non-O1 non-O139 *V. cholerae* were isolated from all sediment, plankton and water samples from the three test sites on each occasion (Tables 4, 5). In contrast the isolation of *Aeromonas* spp. was more variable with isolation rates of 66%, 77% and 55% from sediment, plankton and water respectively (Tables 4, 5) the majority of isolates were *Aeromonas hydrophila* although *Aeromonas sobria* and *Aeromonas caviae* were also isolated (Table 3).

Samples from sewers

Duplicate swabs were taken from two different drain sites in four areas of Vellore. One swab was plated directly onto media the second was enriched in APW before plating. Non-O1 non-O139 *V. cholerae* were isolated at both sites from all areas with the exception of location D where no *V. cholerae* was detected at site one even after enrichment (Table 6).

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Table 4. *Organisms isolated from water bodies*

	Sediment			Plankton			Water		
Site/date	NAG vibrio*	*Aeromonas* spp.	Others	NAG Vibrio	*Aeromonas* spp.	Others	NAG vibrio	*Aeromonas* spp.	Others
Otteri									
17/7	+	+	NFGNB†	+	+	*E. coli* NFGNB	+	+	*E. coli* *Enterobacter* spp.
22/7	+	–	–	+	+	NFGNB *Plesiomonas* spp	+	–	NFGNB *Plesiomonas* spp.
25/7	+	–	–	+	+	–	+	+	NFGNB
Thankal									
17/7	+	+	–	+	+	–	+	–	–
Ondra									
22/7	+	+	NFGNB	+	+	NFGNB	+	+	–
25/7	+	+	–	+	+	–	+	+	*Klebsiella* spp.
Moat									
18/7	+	+	–	+	–	NFGNB	+	–	–
22/7	+	+	NFGNB	+	–	–	+	–	–
25/7	+	–	–	+	+	*E. coli*	+	+	*Enterobacter* spp.

* NAG, non-O1, non-O139 *V. cholerae*.
† NFGNB, non fermenting Gram-negative bacillus.

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Table 5. *Vibrio and aeromonas isolated from drinking water and the environment*

	Drinking water	Environment		
		Sediment	Plankton	Water
NAG* *V. cholerae*	41·6†	100	100	100
Aeromonas spp.	58·3	66·6	77·7	55·5

NAG, non-O1, non-O139 *V. cholerae*.
Values are % of isolates.

Table 6. *Organisms isolated from open drainage*

		Site 1			Site 2		
Location	Date	NAG* *Vibrio cholerae*	*Aeromonas* spp.	Others	NAG *Vibrio cholerae*	*Aeromonas* spp.	Others
A‡	25/7/96	−	+	−	+	+	−
A§		+	+	NFGNB†	+	−	NFGNB
B‡	29/7/96	−	−	*E. coli*	−	+	−
B§		+	+	−	+	+	−
C†	29/7/97	−	−	*E. coli*	−	+	NFGNB
C§		+	+	−	+	+	NFGNB
D‡	29/7/96	−	+	NFGNB	+	+	NFGNB
D§		−	+	−	+	+	NFGNB

* NAG, non-O1, non-O139 *V. cholerae*.
† NFGNB, non fermenting Gram-negative bacillus.
‡ Before enrichment.
§ After enrichment.

Identification of *V. cholerae* O1 and O139 by fluorescent antibody staining

Samples from all three water bodies tested Oteri, Ondra Thankal and the Moat gave a positive reaction for *V. cholerae* O1 and O139 (Fig. 2). In comparison with the positive control the test samples revealed bacteria which appeared rounded up to a greater extent (Fig. 2). False positive results from fluorescent staining can result from auto fluorescence, non-specific binding of the conjugate or cross reactivity of the polyvalent sera used with other organisms. Experiments of this type are difficult to control in practice, as it is not possible to establish a true negative control because the precise composition of the environmental water samples are unknown, and could in theory affect the sensitivity of the test. However, the specificity of the test under defined conditions was confirmed. Positive and negative controls consisted of standard strains of *V. cholerae* O1 and O139, *Escherichia coli*, *Pseudomonas aeruginosa*, non-O1, non-O139 *V. cholerae* clinical isolates and in particular representative strains of *Aeromonas* spp. and non-O1, non-O139 *V. cholerae* which had been isolated from the test samples during the course of the survey. None of the negative controls showed fluorescence confirming the specificity of the test in distilled water and leading to the conclusion that *V. cholerae* O1 and O139 were present in the environmental samples. The possibility however that there were substances in the environmental water interfering with the staining process and affecting the sensitivity of test cannot be completely discounted.

Presence of the *ctx* operon

None of the non-O1, non-O139 *V. cholerae* environmental isolates contained the *ctx* operon.

Antibiotic resistance

The non-O1, non-O139 *V. cholerae* isolates demonstrated a heterogenous pattern of antibiotic resistance

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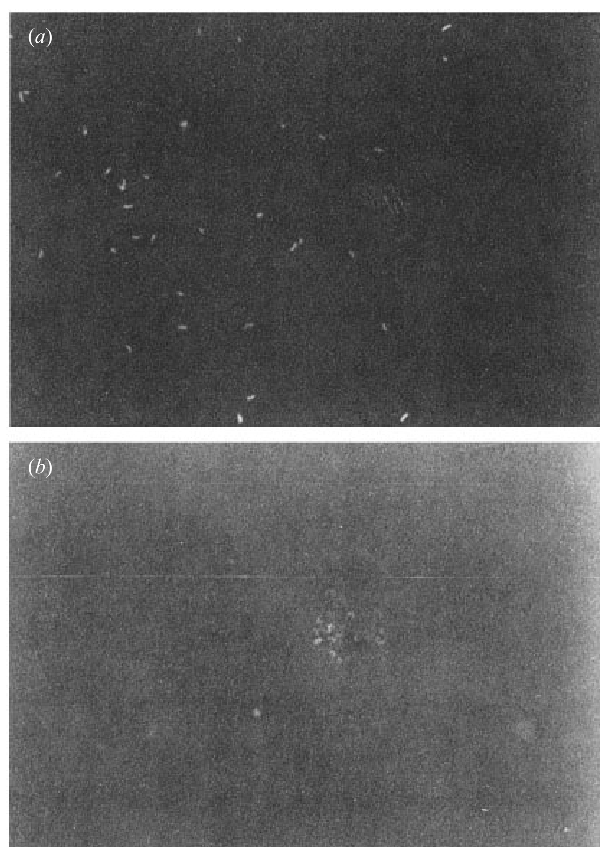

Fig. 2. Fluorescent antibody staining with O139 antibody of samples from the Moat sample site. A, O139 positive control; B, Moat sample.

(Table 7) with resistance to amoxycillin being the most prevalent and variable. Seventeen of the isolates had MICs in excess of 8 mg/l with the range of MICs between 2 and > 256 mg/l. All the isolates were sensitive to ciprofloxacin, chloramphenicol and tetracycline. A single isolate showed high level resistance to trimethoprim.

Table 7. *Antibiotic resistance patterns of non-O1, non-O139* V. cholerae *environmental isolates from Vellore*

Strain	Mic*				
	Amox	Cip	Cm	Tet	Tp
359	4	0·016	1	0·5	2
360	128	0·06	8	0·5	> 256
361	> 256	0·016	1	0·5	2
362	4	0·008	1	0·5	2
363	4	0·008	1	0·5	2
364	4	0·008	1	0·5	2
365	256	0·008	1	0·5	2
366	> 256	0·008	1	0·5	2
367	32	0·008	1	0·5	2
368	4	0·016	1	0·5	2
369	4	0·008	1	0·5	2
370	4	0·016	1	0·5	2
371	256	0·008	1	0·5	2
372	128	0·008	1	0·5	2
373	> 256	0·008	1	0·5	2
374	4	0·008	1	0·5	2
375	2	0·008	0·25	0·5	2
376	64	0·016	1	0·5	2
377	4	0·016	1	0·5	2
378	> 256	0·008	1	0·5	2
379	128	0·008	1	0·5	2
380	2	0·008	0·25	0·5	2
381	> 256	0·016	1	0·5	2
382	2	0·008	1	0·5	2
383	4	0·008	1	0·5	2
384	4	0·12	1	0·5	2
385	4	0·008	1	0·5	2
386	256	0·008	1	0·5	2
387	4	0·004	0·25	0·5	2
388	16	0·008	1	0·5	2
389	2	0·008	0·25	0·5	2
390	16	0·008	1	0·5	2
391	2	0·008	0·25	0·5	2
392	2	0·008	0·25	0·5	2
393	2	0·008	0·25	0·5	2
394	2	0·008	0·25	0·5	2
395	4	0·008	1	0·5	2
396	> 256	0·008	1	0·5	2
397	64	0·016	1	0·5	2
398	4	0·016	2	1	2

* Amox, amoxycillin; Cip, ciprofloxacin; Cm, chloramphenicol; Tet, tetracycline; Tp, trimethoprim.

DISCUSSION

V. cholerae of serotypes O1, O139, a variety of other serotypes and *Aeromonas* spp. were all isolated from patients displaying the symptoms of cholera in Vellore town in the period leading up to and during the study. However, little has been known about the distribution of these strains in the environment and the extent of any water contamination.

Non-O1, non-O139 *V. cholerae* was isolated from all three environmental sites. This is similar to other studies which have demonstrated the ubiquitous nature of these organisms and led to the hypothesis that they, in some way, have a survival advantage over *V. cholerae* O1 and O139 [12–16]. The reservoirs or sites of survival and multiplication of O1 between epidemics are not known, and the low levels of isolation of *V. cholerae* O1 from the aquatic environment by culture techniques, even during outbreaks of cholera infection, has until recently led to the conclusion that *V. cholerae* O1 is unable to survive for long periods in the aquatic environment. However,

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the hypothesis that *V. cholerae* O1 can exist in a non-culturable but viable state in the environment and its possible associations with other water organisms demonstrates that the organism can adapt to changing conditions and this has significant implications for its survival and for its epidemiology [6, 7, 8]. Although most published studies of *V. cholerae* O139 in environmental samples have relied on culture methods it has been postulated that *V. cholerae* O139 can also exist in a viable non-culturable form and that this may account for its pattern of decreasing isolation from the environment following outbreaks. In Vellore although no culturable O1 and O139 strains were detected in the environmental isolates fluorescent antibody staining detected both serotypes in all areas although it is not clear if the organisms detected were in a viable form. Between 1992 and 1996 there were widespread clinical cholera cases caused by both these serotypes in Vellore [4, 5]. It is not clear if this led to the dissemination of these strains into the environment leading to their detection in this study or indeed if the opposite was true and the appearance of these serotypes in the environment led to increased clinical disease. It has been speculated that the introduction of toxigenic strains of *V. cholerae* O1 and O139 strains into the environment leads to an increase in toxin production amongst the other aquatic serotypes by means of genetic exchange [14]. In this survey none of the non-O1, non-O139 isolates of *V. cholerae* contained the *ctx* operon as determined by PCR suggesting that this has not occurred in Vellore. However it is interesting to note that some of these strains show toxigenic effects when tested on Vero and CHO cells lines indicating the occurrence of other toxins (Balaraman and Jesudason, unpublished results). Although not used for drinking water all the sample sites are used for a variety of purposes including bathing and washing clothes and utensils; activities which have been demonstrated to be associated with increased risk of contracting cholera even if culture-negative water is used for drinking [17]. In addition, the moat is also used for fishing. *Aeromonas* spp. are ubiquitous aquatic organisms and their isolation from the environmental sites was not unusual.

Non-O1, non-O139 *V. cholerae* and *Aeromonas* spp. were isolated from drinking water samples. Interestingly, no isolates were obtained from the sample taken immediately after water had left the treatment plant suggesting that contamination was not occurring at source but at some point within the piped supply to the town. The viable organisms recovered, may be themselves diarrhoeagenic but, in addition, it demonstrates the potential for the supply to be a source of infection during outbreaks caused by O1 or O139 serotypes. The two most common species of *Aeromonas* isolated from the drinking water, *Aeromonas hydrophila* and *Aeromonas sobria* are the commonest clinical isolates of *Aeromonas* spp. In Vellore where a typical distribution of clinical isolates would be *A. hydrophila* 50%, *A. sobria* 40% and *A. caviae* 10% (Jesudason, unpublished results). Water supplies tested were from communal stand pipes in the defined areas of the town to which water is pumped at set times during the day. The stand pipes are often in close proximity to open sewerage channels giving the potential for cross contamination. Samples taken from sewerage channels beside water stand pipes were culture positive for non-O1, non-O139 *V. cholerae* and *Aeromonas* spp.

It has been speculated that antibiotic resistance may have a role in the selection and spread of new clones of toxigenic *V. cholerae* serotypes. One of the characterizing features of *V. cholerae* O139 when it emerged was its characteristic resistance pattern including resistance to trimethoprim-sulphamethoxazole and it appears that in some cases this results from the presence of a conjugative transposon [18]. The environmental isolates in this study showed a heterogenous antibiotic resistance pattern. Although in contrast to clinical isolates of *V. cholerae* O1 and O139 in Vellore there was almost uniform sensitivity to trimethoprim. The high levels of ampicillin resistance were unexpected.

An understanding of the aquatic environment holds the key to the events surrounding the emergence of *V. cholerae* O139 and hence the likelihood of this happening again. The sources and routes of cholera infection in Vellore were not clear. In this first study we have demonstrated the contamination of the water supply with non-O1, non-O139 *V. cholerae* and although not detectable by culture we have also shown that both *V. cholerae* O1 and O139 serotypes were widespread in the environment in Vellore. Further studies are planned to investigate how the environmental distribution alters throughout the year and relates to the clinical pattern of cholera in Vellore during extensive outbreaks.

https://doi.org/10.1017/S0950268898001010 Published online by Cambridge University Press

ACKNOWLEDGEMENTS

We should like to thank the Leverhume Trust for grant number R05847 for funding the consumables for this project and the associated field visit to India and Bayer plc for funding the Domagk Lectureship awarded to C.J.T.

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