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Ecological study of *Vibrio cholerae* in Vellore

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SUMMARY

Vellore is endemic for cholera due to *Vibrio cholerae* O1 and O139. In a previous study the prevalence of *Vibrio cholerae* in drinking water, lakes and sewage outfalls in a single 2-months period in Vellore, India was determined. In addition water samples from three sites were also tested for the presence of *V. cholerae* O1 and O139 by fluorescent antibody staining. This follow on study has examined how the environmental distribution of *V. cholerae* at the same sites alters over a 12-month period and the relationship to the clinical pattern of cholera in Vellore. Samples of water were collected from fixed sites at three water bodies each month between April 1997 and March 1998. 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. Samples were also tested for the presence of *V. cholerae* O1 and O139 by fluorescent antibody staining. The clinical isolation rate of *V. cholerae* in Vellore, maximum temperature and rainfall were also studied. The results demonstrate the presence in the environment of viable but non-cultivable (VNC) *V. cholerae* in 10 of 12 months of the study year as well as their viability. Their prevalence in the environment also correlated with the isolation of these pathogens from clinical samples over the same study period.

INTRODUCTION

Vellore is endemic for cholera and *V. cholerae* is isolated from patients throughout the year with usually two seasonal increases in isolation in April–May and September–October. Since 1991 when *V. cholerae* serogroup O139 emerged as a choleragenic vibrio [1], we have observed, in Vellore, a change in the relative prevalence pattern of serogroups O1 and O139 [1–3]. The reason for this fluctuation in the clinical pattern of cholera in Vellore are not clear but may result from environmental factors. It is therefore important to ascertain the environmental niche of these organisms during the dormancy period. Previously it had been difficult to correlate environmental distribution of *V. cholerae* and outbreaks of clinical disease. However, the observation that both *V. cholerae* serogroups O1 and O139 were known to enter a viable though non-cultivable (VNC) phase in the environment [4]. May help us understand better the environmental distribution of *V. cholerae* and its routes of transmission. A previous study undertaken in July/August 1996 to provide a baseline for future studies demonstrated that although no cultivable O1 and O139 strains were detected in the three main sample sites around the town, fluorescent antibody staining detected both serotypes in all areas. The aim of this study was to extend the work previously reported by examining how the environmental dis-

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tribution of *V. cholerae* O1 and O139 altered throughout the year. Furthermore our aim was to investigate the relationship, if any, of this to the clinical pattern of disease in Vellore and other environmental factors such as rainfall and temperature.

METHODS

Source of samples

Samples were collected in and around the town of Vellore which is located 120 km west of Madras, South India between April 1997 and March 1998. Samples of surface water were collected from three water bodies in and around Vellore which had been studied previously [5].

Two of the sample sites, Otteri and Katpadi (Ondranthankal) were freshwater pools located at 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 a freshwater 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 sold in the local market.

Sample collection and processing

Water samples were collected as previously described [5] from each site once per month between April 1997 and March 1998. Water was collected 2–3 m away from the bank and at a depth of 30 cm in sterile flask. Flasks were held at the bottom and the mouth immersed to a depth of 30 cm in the water. The samples was filtered through 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 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 containing APW. 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. The APWs were incubated at 37 °C for 6 h and subsequently subcultured onto solid media as described previously [5]. Clinical samples of stools from patients suspected of having cholera were processed by routine methods as described previously [3]. Identification of isolates was performed by standard biochemical tests [5]. After biochemical confirmation all *V. cholerae* were serologically confirmed as well with polyvalent O1 and O139 antisera in slide agglutination tests [5].

Detection of *V. cholerae* O1 and O139 in the environment using fluorescent antibody

Immunofluorescence was performed by a modification of the method described by Xu and colleagues [6], using antiserum, polyvalent O1 or O139 supplied from the National Institute of Cholera and Enteric disease, Calcutta. Fluorescein-labelled conjugate polyclonal anti-rabbit IgG, developed in goat, obtained from Sigma, Dorset, UK was also used. In order to quantify the presence of O1 and O139 vibrios the results of immunofluorescence were arbitrarily quantified as 10/20/30 fluorescing bacilli per high power field of view.

Viability studies

Viability studies were carried out on the water samples positive for *V. cholerae* O1 and O139 by IFA. One-millilitre aliquots of the positive water samples were cultured in yeast extract with nalidixic acid for 6 h at 25 °C. Subsequently fixed smears were stained by Gram stain as well as treated with *V. cholerae* O1/O139 antiserum and then FITC conjugate [7].

Environmental factors

Maximum monthly temperature and rainfall data for Vellore during the study period were obtained from the regional meteorological centre, Nungambakkam, Madras.

RESULTS

Isolation from clinical samples

Strains of *V. cholerae* O1 were isolated from patients each month ranging between 1 and 40 % during the study period with a peak in January 1998 with 40 %. Fewer clinical isolates *V. cholerae* O139 were obtained during the study period with peak incidences in

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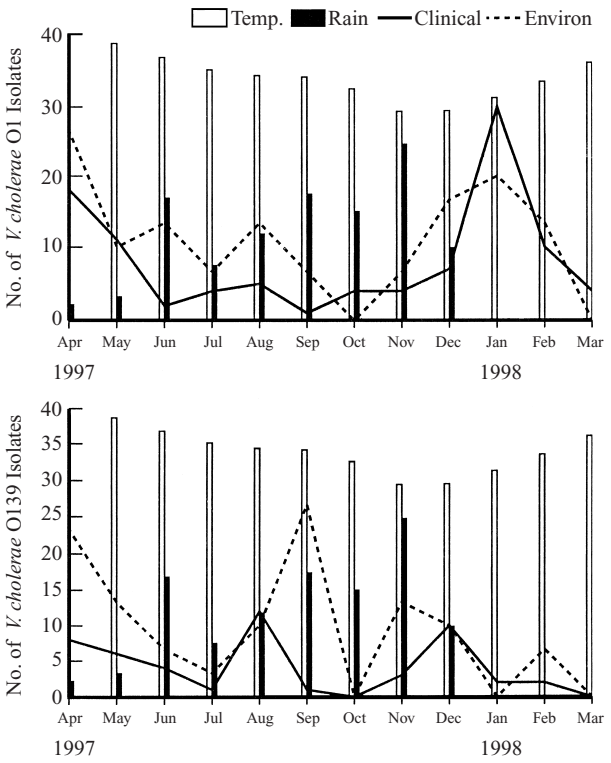

No. of the environmental strains each month is a mean of isolations from the three sites

Fig. 1. Monthly isolation of *V. cholerae* O1 & O139 from clinical specimens and environmental sources in relation to temperature and rainfall.

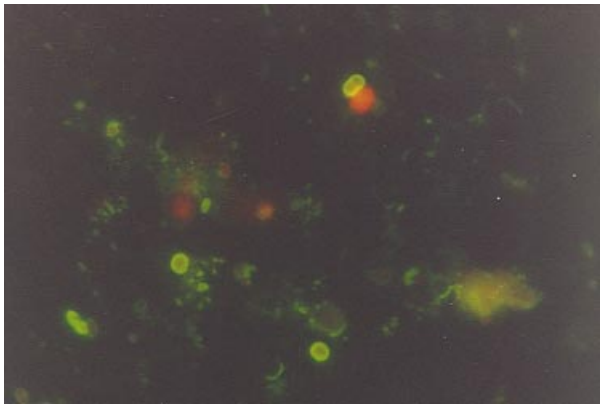

Fig. 2. Immunofluorescent staining of dormant forms of *Vibrio cholerae* O1. Magnification, × 500.

August 1997 and December 1997. Figure 1 shows this data as well as the pattern of isolation of vibrios from the environment.

Immunofluorescence studies

Both *V. cholerae* O1 and O139 strains were detected during study period. Morphologically typical bacillary forms as well as rounded forms (doughnuts) as described by earlier workers [7] were seen for both

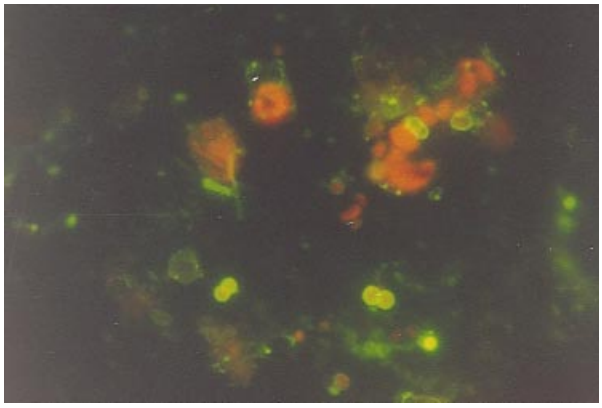

Fig. 3. Immunofluorescent staining of dormant forms of *Vibrio cholerae* O139. Magnification, × 500.

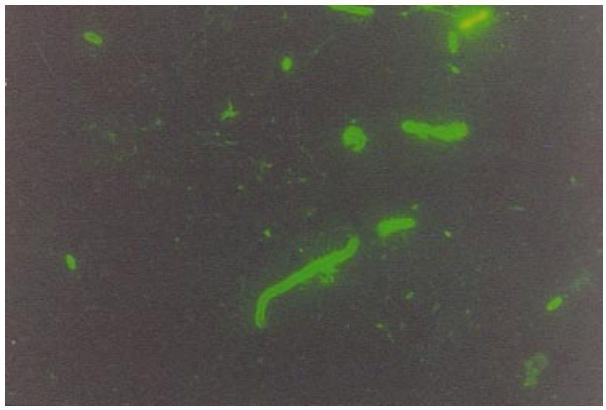

Fig. 4. Immunofluorescent staining of elongated forms with no bacterial multiplication. Magnification, × 500.

serogroups (Figs 2, 3). VNC forms of *V. cholerae* O1 were detected each month with the exception of October 1997 and March 1998. VNC *V. cholerae* O139 was detected each month except October 1997, January and March 1998. Both *V. cholerae* O1 and O139 VNC forms were highest during April 1997. On the whole, *V. cholerae* O1 was detected in a slightly higher percentage than O139 during the study period from all three water bodies, Otteri, Katpadi (Ondranthankal) and moat. Of the three water bodies, the highest isolation of *V. cholerae* O1 and O139 was from Katpadi (Ondranthankal) (Tables 1, 2).

Viability studies

All samples positive for *V. cholerae* O1 and O139 by IFA were tested for viability and were observed as elongated bacillary forms with no bacterial multiplication (Fig. 4). All samples tested were positive in the viability study.

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Table 1. *Detection of viable non-cultivable vibrios from three environmental sites. April 1997 to March 1998 by immunofluorescence technique*

		O1		O139	
Month	Location	Presence*	Grading†	Presence*	Grading†
April	Katpadi	+	20	+	20
	Moat	+	30	+	30
	Otteri	+	30	+	20
May	Katpadi	−		+	10
	Moat	−		+	10
	Otteri	+	30	+	20
June	Katpadi	+	10	−	
	Moat	+	10	−	
	Otteri	+	20	+	20
July	Katpadi	−		+	10
	Moat	−		−	
	Otteri	+	20	−	
August	Katpadi	+	20	+	30
	Moat	−		−	
	Otteri	+	20	−	
September	Katpadi	+	10	+	30
	Moat	+	10	+	30
	Otteri	−		+	20

* +, positive; −, negative.
† Grading: fluorescing bacilli per high power field.

Table 2. *Detection of viable non-cultivable vibrios from three environmental sites. April 1997 to March 1998 by immunofluorescence technique*

		O1		O139	
Month	Location	Presence*	Grading†	Presence*	Grading†
October	Katpadi	−		−	
	Moat	−		−	
	Otteri	−		−	
November	Katpadi	+	20	+	10
	Moat	−		+	10
	Otteri	−		+	20
December	Katpadi	+	30	−	
	Moat	+	20	−	
	Otteri	−		+	30
January 1998	Katpadi	+	30	−	
	Moat	+	30	−	
	Otteri	−		−	
February	Katpadi	+	10	+	10
	Moat	+	20	−	
	Otteri	+	10	+	10
March	Katpadi	−		−	
	Moat	−		−	
	Otteri	−		−	

* +, positive; −, negative.
† Grading: fluorescing bacilli per high power field.

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Environmental factors

The average monthly temperature and rainfall during the period of the study in relation to isolation of vibrios from clinical samples and environment sources is shown in Figure 1. The peak temperature recorded was 38·7 °C in May 1997 and maximum rainfall was 24·98 mm in November 1997.

DISCUSSION

Since 1991 when *V. cholerae* serogroup O139 emerged as a vibrio, we have observed in Vellore a change in the relative prevalence pattern of serogroups O1 and O139 [1–3]. In such a situation we felt that it is worthwhile ascertaining the environmental niche of this organism during the dormancy period. Although the precise role of the natural environment in the transmission and epidemiology of cholera is not clear, natural water bodies have been implicated both as sources and reservoirs of *V. cholerae* [8].

Working on the hypothesis that *V. cholerae* O1 can exist in a viable but non-cultivable state in the environment, our study group had earlier demonstrated that in Vellore although no cultivable O1 and O139 strains were detected in the environment, fluorescent antibody staining detected both serogroups in water bodies at specific times of the year [5]. In the present study both *V. cholerae* serogroups were detected by IF in majority of the water samples throughout the study period. Fluorescent comma-shaped bacilli and doughnut forms were detected. Baker and colleagues [9] have reported these as representing vibrios in a state of dormancy. To implicate these VNC forms in disease causation, their viability needs to be established. We have demonstrated this in the present study as described by Kogure and colleagues 1979 [7] in which active cells are identified by growth without multiplication in response to the addition of yeast extract in the presence of nalidixic acid. Under these conditions, active cells carry out protein synthesis in the absence of DNA replication or cell division and produce elongated cells. This was observed in samples from all three water bodies. This could suggest that these three water bodies, among possibly others are sources and reservoirs for cholera in Vellore town. Human volunteer studies suggested that VNC *V. cholerae* maintains pathogenic potential in the environment with human passage effectively triggering an outgrowth of the cells to the viable and cultivable state [10]. *V. cholerae* from human cases/carriers spreads in the environment and survives and multiplies under favourable conditions and assumes dormant forms until these return.

We have examined the environmental distribution over 1 year and compared it with clinical isolation pattern of *V. cholerae* over the same time-frame. No seasonality was observed in clinical isolation. It is our observation that increased appearance of the serogroups in the environment coincides with the clinical isolation pattern. *V. cholerae* O1 detection in the environment peaked in April and August 1997 and January 1998 which coincided with an increase in isolation from clinical samples; likewise with *V. cholerae* O139 in April, August–September and November–December of 1997, and February 1998.

During the 1-year study period, no correlation was observed in isolation pattern of both serogroups and maximum temperature recorded in Vellore. The peak incidence of clinical cholera due to *V. cholerae* O1 seemed to follow the highest rainfall recorded but this was not so with *V. cholerae* O139. In Vellore although no cultivable O1 and O139 strains were detected in the environmental sources, fluorescent antibody staining detected dormant forms of both serogroups in the three water bodies and it seems that these dormant forms were in a viable state. Between 1992 and 1998 there were widespread clinical cholera cases caused by both these serogroups in Vellore. It is still not clear if this latter led to the dissemination of these strains into the environment leading to their detection in this study or whether appearance of these serogroups in the environment precedes the increase in the clinical isolation.

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