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Detection of *Vibrio cholerae* O1 in the Aquatic Environment by Fluorescent-Monoclonal Antibody and Culture Methods

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***Vibrio cholerae* O1 in plankton samples collected from ponds and rivers between February 1987 and January 1990 in Matlab, Bangladesh, was detected by the fluorescent-monoclonal antibody (FA) technique. Samples were collected at sites which were monitored fortnightly (fixed sites) as well as at sites that were part of a case-control study. FA results were compared with those obtained by conventional culture methods (CM). A total of 876 samples were collected; *V. cholerae* O1 was detected in 563 samples (64.27%) by the FA method and in 3 samples (0.34%) by CM. Of the fixed-site plankton samples, 439 (63.62%) were positive by FA and none were positive by CM. Of the 93 case sites sampled on the day after the occurrence of a case of cholera, 73 (78.49%) were positive for *V. cholerae* O1 by FA and 3 (3.2%) were positive by CM. In comparison, of the 93 first-day sample collections at control sites at the time a case of cholera occurred, only 51 (54.83%) were positive by FA and none were positive by CM. From the data, it is concluded that *V. cholerae* O1 is present throughout the year in the ponds and rivers of Bangladesh that were examined in this study and that *V. cholerae* can be detected by FA but not always by CM. The FA procedure was found to be very useful in detecting *V. cholerae* in plankton, with which it was associated and often occurred in large numbers in the nonculturable stage. Thus, studies investigating the significance of the role of environmental factors in the epidemiology of cholera can be performed effectively by using FA. Such studies are in progress.**

Vibrio cholerae O1 is recognized as the etiological agent of pandemic Asiatic cholera. Environmental reservoirs for this agent of ravaging disease have been sought (9), and natural bodies of water are known to serve as both reservoirs for the organism and means of its transmission to humans. However, isolation of *V. cholerae* from lakes, rivers, streams, and ponds in epidemic areas has not always been successful (2). Studies have shown that *V. cholerae* can be found associated with aquatic hydrophytes (7) and zooplankton (5). Laboratory microcosm experiments have documented attachment, survival, and proliferation of *V. cholerae* O1 on copepod surfaces (5). Furthermore, investigators have detected *V. cholerae* in aquatic samples collected during epidemics, but the organism usually cannot be cultured from aquatic environments during interepidemic periods. The existence of nonculturable but still viable cells in aquatic environments has been postulated (17).

Bangladesh is a country in which cholera is epidemic, and the occurrence of cholera outbreaks demonstrates a predictable seasonal pattern, with the highest frequency of outbreaks occurring between August and December (4). During interepidemic periods, *V. cholerae* O1 is not readily isolated from water samples by conventional culture methods that employ an enrichment step with an alkaline peptone water medium followed by plating on tellurite-taurocholate-gelatin agar or thiosulfate-citrate-bile salts-sucrose agar. Results of laboratory microcosm studies showed that *V. cholerae* O1 cells exposed or adapted to such environmental conditions as low nutrient concentrations in the growth medium and low temperature are unable to grow or multiply in nutrient-rich media at elevated temperatures (11, 16). The fluorescent-monoclonal antibody (FA) staining procedure has been used successfully for direct detection of viable but not culturable *V. cholerae* O1 in environmental water samples. Results of such studies showed the presence of higher *V. cholerae* O1 counts than could be obtained by conventional culturing methods (1, 17).

Having reported the association of *V. cholerae* O1 with aquatic plankton, i.e., both phytoplankton and zooplankton, and attachment of *V. cholerae* to surfaces of zooplankton, e.g., copepods (5), we concluded that water serves as a reservoir for this organism. The study reported here was designed to demonstrate both the occurrence of *V. cholerae* in water and its seasonal distribution in plankton samples. Water sources were hypothesized as more likely to have some kind of association with clinical cases. To test this hypothesis, a fluorescence-labeled monoclonal antibody was employed in the FA technique discussed elsewhere (1), a method shown to be highly specific for detection of *V. cholerae* O1 in water samples.

MATERIALS AND METHODS

Fixed sites and samples. Samples were collected from 10 fixed sites in Matlab, which is located 45 km southeast of Dhaka, Bangladesh, in the delta formed by the Meghna and Ganges rivers. Fixed sites are those which were sampled on a regular basis throughout the study. From these 10 sites, which included eight ponds and two river stations, water and plankton samples were collected at 14-day intervals after sunset from February 1987 through January 1990. Water samples for bacteriological studies were collected in presterilized 125-ml glass bottles. Plankton samples were collected by filtering 50 liters of water through a plastic sampler fitted with a 0.77-μm-mesh net, achieving a final concentration of 1,000 times the original concentration. From the concentrated plankton sample, which had a final volume of 50 ml, 10

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ml was transferred into each of three vials directly after sampling, and while still in the field these samples were preserved in formaldehyde at a final concentration of 4%. Studies done previously at these sites in Bangladesh have documented that the FA counts and direct viable counts are approximately the same (1). In this study, for direct detection of *V. cholerae* O1, only the FA method was employed. Of the remaining 20 ml of each sample, 10 ml was homogenized with a Teflon-tipped tissue grinder (StedFast Stirrer, model 300; Fisher Scientific Co., Pittsburgh, Pa.) and enriched by addition of alkaline peptone broth for isolation of *V. cholerae* by conventional culture methods (15).

Case-control sites. In addition to those sites sampled every 2 weeks, samples were collected each time a case of cholera was reported at the Matlab Treatment Center during the 3-year study period. Water and plankton samples were collected the day after the case was reported and once a week for 3 weeks thereafter from the pond or any other source of water used by that particular cholera victim. Each time a case of cholera occurred, a control site was also selected for the study. These control sites were ca. 1 to 2 km away from the site where the case of cholera occurred, ecologically similar to the water source used by the cholera patient, and from an area where there were no current reports of diarrheal cases. Data reported in this article are for samples collected the day after the case occurred from the control pond and from the case pond or any other water sources used by the patient at the time he or she became ill. A total of 93 cases were investigated during the 3-year study reported here.

FA technique. For detection of *V. cholerae* O1, formaldehyde-preserved plankton samples were processed by a technique described by Brayton et al. (1). One milliliter of sample was placed in a 1-dram (ca. 3.697-ml) plastic vial constructed with a 0.77-μm-mesh net at the bottom. The vials with the plankton samples were placed in 16-mm wells of tissue culture plates for washing and challenging with monoclonal antisera. After exposure to antibody, the samples were washed in 0.1 M phosphate-buffered saline (pH 7.4). As a positive control for the staining procedure, one of the vials containing plankton was inoculated with *V. cholerae* O1 (ATCC 14035 or ATCC 14033), and as negative controls, various strains of members of the families *Enterobacteriaceae* and *Vibrionaceae* (other than *V. cholerae* O1) were included in the procedures.

After incubation, the suspensions were again washed with phosphate-buffered saline, and a monoclonal antibody specific to the A antigen of the O1 lipopolysaccharide, pretested for specificity (1), was used at a concentration of 10 μl/ml in phosphate-buffered saline and preserved by the addition of gentamicin. The mixture was incubated for 1 h at 35°C.

Anti-mouse immunoglobulin G-conjugated fluorescein isothiocyanate (Sigma Chemical Co., St. Louis, Mo.) was added to the vials to a final concentration of 10 μl/ml. Samples were maintained in the dark at room temperature during preparation of slides and observation of the stained preparations with an epifluorescence microscope (model BH-2; Olympus). Cells of *V. cholerae* O1 appeared as small, distinct rods surrounded by a bright fluorescent green band which was dramatically evident, as in the color photograph published by Tamplin et al. (Fig. 1 in reference 13).

Statistics. The percentages of FA-positive plankton samples in case and control sites were compared by the McNemar test (12). Similar analyses were performed to compare plankton samples tested with FA with samples tested by conventional culture methods. Since all these results were highly significant ($P \leq 0.01$), no attempt was made to adjust for multiple comparisons.

TABLE 1. Detection of *V. cholerae* O1 in fixed sites and case-control study water sources

Source (no.) of samples	No. (%) of samples positive for *V. cholerae* by[a]:		
	FA method (plankton samples)	Culture method	
		Plankton samples	Water samples
Fixed site (690)	439 d (63.62)	0 a	6 a (0.87)
Case site (93)[b]	73 cd[c] (78.49)	3 a (3.2)	4 a (4.3)
Control site (93)[b]	51 cd[c] (54.83)	0 a	0 a
Total (876)	563 (64.27)	3 (0.34)	

[a] Values in the same row that are followed by the same letter are not significantly different (McNemar test; $P \leq 0.01$). Note that all samples positive by conventional culture methods were also positive by the FA method.

[b] Samples obtained the day after the report of a case of cholera.

[c] Case and control sites were significantly different by the McNemar test ($P \leq 0.01$).

The data collected biweekly at the 10 fixed sites were analyzed by first computing the percentage of samples positive by the FA method for each month by site combination. An arcsine transformation was applied, and the resulting data were analyzed in a two-way analysis of variance with site and month as the effects in the model. Model assumptions were determined by examining residuals for normality (12).

RESULTS AND DISCUSSION

Of 690 samples collected from the established sampling sites, *V. cholerae* O1 was detected in 439 (63.62%) of the plankton samples by the FA technique (Table 1). All plankton samples, however, remained negative for *V. cholerae* O1 by conventional culturing methods, and *V. cholerae* O1 was isolated from only six water samples by conventional culture methods.

Of 93 case sites (the sites where cholera cases had occurred in a village and the samples taken from the pond or river that served as the source of water for that patient) and 93 control site samples (collected at the same time as the case of cholera was recorded), 73 (78.49%) and 51 (54.83%), respectively, were positive for *V. cholerae* O1 by the FA method (McNemar test; $P \leq 0.01$) (Table 1). Four water samples and three plankton samples collected at the case sites were culture positive, whereas none of the water or plankton samples collected at the control sites were positive by culture methods. In both case and control sites, the FA method was successfully employed to detect a significantly higher number ($P \leq 0.01$) of samples positive for *V. cholerae* O1 than that detected by conventional culture.

The range of distribution of *V. cholerae* O1 in fixed-site samples, measured by the FA technique, is shown in Fig. 1. As can be seen in this figure, the range of percentages of samples positive by the FA method for each month with data collected over a 3-year period indicates that the months of March and April had the lowest mean number of FA-positive samples for *V. cholerae* O1 in samples collected at the fixed sites, while the months of August through November had the highest number of positives. It is evident that *V. cholerae* O1 was present in the water and on plankton throughout the year but that significant differences could be found among months ($P \leq 0.01$) and sites ($P \leq 0.01$). Similar observations were made for samples collected from the case-control study. The epidemiological significance of this finding is being analyzed further, and results will be published elsewhere.

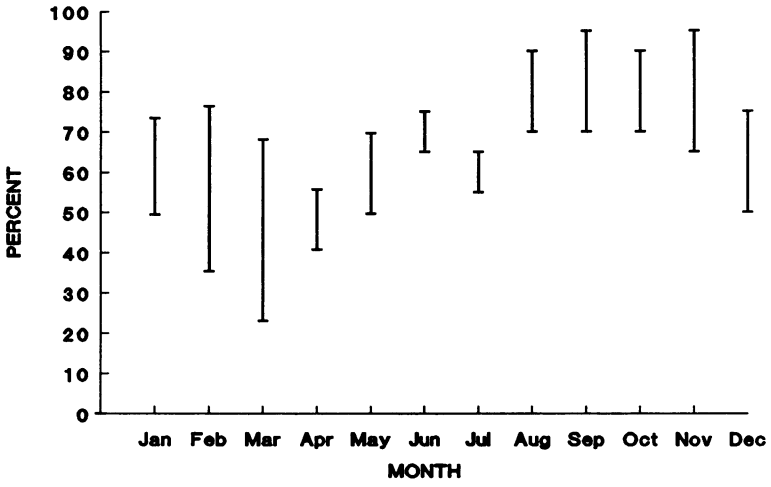

FIG. 1. Range of distribution of *V. cholerae* O1 detected by the FA method over a period of 3 years (February 1987 through January 1990).

One of the fixed sites, a pond within a religious territory and excluded from daily use, was originally included in the study because it was ostensibly protected from human use. However, 46 (65.71%) of the 71 analyzed samples from this pond were positive for *V. cholerae* O1 when tested by the FA method. None of the samples, however, was positive by the culture procedures.

V. cholerae O1 was found to colonize both phytoplankton and zooplankton surfaces. Colonization by *V. cholerae* O1 cells was observed to be most extensive for copepods. Colonization occurred often on other planktonic species as well, such as *Rotifera*, *Cladocera*, *Volvox*, *Daphnia*, and *Desmida* spp. This observation confirms findings reported previously (13). Colonization by *V. cholerae* O1 of intact plankton and fragments of plankton species was also detected by the FA method.

The FA technique has been shown previously to be useful in detecting viable but nonculturable *V. cholerae* O1 in water samples (13, 17). Results of several studies have shown that various biological and physicochemical factors influence the growth, survival, and distribution of *V. cholerae* in aquatic environments (3, 8). In addition, ecological relationships of zooplankton and *V. cholerae* O1 have been suggested on the basis of results of laboratory experiments (5, 6). However, the nonculturable but viable forms of *V. cholerae* pose a problem for the accuracy of enumeration of these organisms in the natural environment if only the conventional culture methods are employed.

Venkateswaran et al. (14) recently reported that the incidence of *V. cholerae* non-O1 was below detectable levels in zooplankton populations. This supports our finding that *V. cholerae* is present but is in a viable but nonculturable state, because conventional cultural methods were used to enumerate *V. cholerae*. By combining the FA technique and culture methods, as was done in our study, those factors involved in maintaining the viability and survival of *V. cholerae* in aquatic environments can be elucidated. Thus, in our study, detection of *V. cholerae* O1 at sites sampled regularly at fixed intervals showed that even a pond with restricted human use yielded evidence of the presence of *V. cholerae* O1 when tested by the FA method. This control pond is located within the compound of a sacred place and is not open for routine human activity, nor does it possess any inlet from any other water supply. Samples collected from this pond consistently gave negative results by culture methods, just as samples collected from control sites of the case-control study did.

It is evident that the findings reported here are significant in that they demonstrate the presence of *V. cholerae* O1 in waters of regions in which cholera is endemic as well as a statistically significant seasonal shift in abundance of *V. cholerae* O1 in the environment. Nonculturable but viable *V. cholerae* O1 may offer an explanation of the mode of transmission of *V. cholerae* and the historically difficult task of demonstrating the presence of this organism in water. Direct FA staining, coupled with microscopic observation, makes it possible to detect these viable but nonculturable *V. cholerae* O1 even during interepidemic periods in areas where cholera is endemic when all conventional methods of culturing fail.

Results of studies reported in the literature appeared to suggest that the association of *V. cholerae* with plankton is confined to copepods and a few of the aquatic hydrophytes (6, 13). In this study, we were able to observe the attachment of *V. cholerae* O1 to a variety of zooplankton and phytoplankton, including *Rotifera*, *Cladocera*, *Daphnia*, *Volvox*, and *Desmida* spp. The most extensive colonization of *V. cholerae* O1 was observed with copepods, but in samples where few copepods were present, other zooplankton or phytoplankton species were also colonized. Thus, *V. cholerae* O1 may alter its association throughout the year as the species composition of the plankton populations in the water column of a given body of water changes. It is possible that the culturable, free-living *V. cholerae* in water attaches to plankton to withstand environmental shifts in temperature, salinity, or nutrient concentration and becomes nonculturable during given periods in response to the environment. Under conditions favorable for growth, *V. cholerae* may convert to the culturable stage, thereby posing an epidemic threat. Such a phenomenon has obvious epidemiological significance if blooms of selected species of plankton are able to trigger a subsequent increase of *V. cholerae* O1 by supporting its outgrowth and multiplication. This hypothesis is now under investigation in our laboratory.

In conclusion, we suggest that *V. cholerae* O1 can be present throughout the year in aquatic environments of areas

where cholera is endemic but that culture methods may fail to achieve isolation and enumeration of the organism, depending on plankton biomass and species composition as well as the physicochemical properties of water and sediment or the physiological state of the *V. cholerae* itself, e.g., whether the cells are actively growing or in a dormant state (10). Molecular probes for *V. cholerae* O1 are being constructed in order to confirm these findings with molecular genetic evidence. In any case, the results of the study reported here indicate that the epidemiology of cholera may be far more dependent upon environmental factors than has been heretofore recognized.

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