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Enumeration of *Vibrio cholerae* O1 in Bangladesh Waters by Fluorescent-Antibody Direct Viable Count

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A field trial to enumerate *Vibrio cholerae* O1 in aquatic environments in Bangladesh was conducted, comparing fluorescent-antibody direct viable count with culture detection by the most-probable-number index. Specificity of a monoclonal antibody prepared against the O1 antigen was assessed and incorporated into the fluorescence staining method. All pond and water samples yielded higher counts of viable *V. cholerae* O1 by fluorescent-antibody direct viable count than by the most-probable-number index. Fluorescence microscopy is a more sensitive detection system than culture methods because it allows the enumeration of both culturable and nonculturable cells and therefore provides more precise monitoring of microbiological water quality.

Vibrio cholerae serovar O1 is the causative agent of epidemic cholera in Bangladesh. Its persistence and transmission are fostered by environmental and climatic conditions and sanitary practices. Frequent exposure of villagers to contaminated surface waters makes it mandatory that improved detection methods for *V. cholerae* be developed to evaluate water quality and assess the risk of disease.

Recent laboratory studies have shown that the organism can exist in a viable but nonculturable state. Furthermore, animal models have demonstrated that such cells can regain culturability and remain pathogenic (1a). As a result of these findings, alternatives to standard culture methods were sought to detect and enumerate nonculturable *V. cholerae* serovar O1.

In this report, we describe a field trial using fluorescent antibody (FA) in direct viable counts (FA-DVC), as described by Brayton and Colwell (1), for detection and enumeration of *Vibrio cholerae* O1 occurring in the natural environment. Fluorescence microscopy was compared with standard culture methods to determine the relative sensitivity and rapidity of the two methods.

MATERIALS AND METHODS

Sample collection. Sampling for the experiments was conducted in January 1986 in villages surrounding Matlab, Bangladesh, where cholera is endemic. The region is located 30 miles southeast of the capital city of Dhaka and lies in the delta formed by the Meghna and the Ganges rivers.

All water samples were collected in sterile containers from village tube wells, ponds, and the adjacent river. Temperature, salinity, and pH were measured with a YSI meter (Yellow Springs Instrument Co., Yellow Springs, Ohio). Initial processing of samples was done at the Matlab laboratory branch of the International Centre for Diarrhoeal Disease Research, Bangladesh within 2 h of collection.

Most-probable-number enumeration. For most-probable-number estimates of *V. cholerae* O1 (3), 100-, 10-, and 1.0-ml samples of water were inoculated into alkaline peptone broth tubes (grams per liter: peptone, 10; NaCl, 10; sodium taurocholate, 5; sodium carbonate, 1 [pH, 8.6]) with three replicates of each volume. Tubes were incubated at 35°C for 6 h, and then a loopful from each tube was streaked onto thiosulfate citrate-bile salts-sucrose agar (Oxoid Ltd., Columbia, Md.). Plates were incubated at 35°C for 18 to 24 h, at which time yellow colonies were picked and subcultured to tryptic soy agar (Difco Laboratories, Detroit, Mich.) amended with 0.5% (wt/vol) NaCl. After incubation at 35°C, slide agglutination tests were performed on all isolates by using *V. cholerae* poly O1 antiserum (Difco). Any isolate yielding a positive test was confirmed by FA staining with monoclonal antibody (see below).

Fluorescence microscopy enumeration. Because the pond water samples examined in our study were relatively turbid, prefiltering through a 1.2-μm-pore-size filter (Gelman Sciences, Inc., Ann Arbor, Mich.) was necessary.

Acridine orange direct counts (AODC) were completed on all samples by the protocol of Hobbie et al. (5). Each sample was Formalin fixed (2% [vol/vol]) and refrigerated until counts were performed.

FA counts for total *V. cholerae* O1 were determined by the method of Xu et al. (21). However, *V. cholerae* monoclonal antibody specific for the A antigen of O1 lipopolysaccharide (M. L. Tamplin, manuscript in preparation) and goat anti-mouse fluorescein isothiocyanate (Difco) were employed as reagents.

FA-DVC of *V. cholerae* O1 were done by the method of Brayton and Colwell (1).

Specificity determination of the monoclonal antibody. To confirm the specificity of monoclonal antibody to *V. cholerae* O1 antigen, FA screening was conducted for a variety of bacterial strains that may be present in environmental samples. All cultures were grown overnight in T_1N_1 broths (1% [wt/vol] tryptone–1% [wt/vol] NaCl) at 25 or 35°C, depending on the requirements of the bacterial strain. Loopfuls of culture were transferred to a tube of phosphate-buffered saline (grams per liter: NaCl, 8.5; Na_2HPO_4, 9.1; KH_2PO_4, 1.5; [pH 7.3]), until a suspension yielding a concentration of 10^6 to 10^7 cells per ml, as determined by AODC, was achieved. At this point, 2.0 ml of the phosphate-buffered saline suspension was filtered through a 25-mm polycarbonate membrane filter with a pore size of 0.2 μm (Nuclepore Corp., Pleasanton, Calif.), and was stained by the FA procedure. Fluorescence microscopy was used to examine 100 fields of each filter to assess cross-reactivity between the monoclonal antibody and the test strains. Bac-

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terial strains included in the study were *V. cholerae* O1 ATCC 14035, ATCC 14033, and 599655; *V. cholerae* non-O1 SG7509, 2030H, SG8268, N8246, 42, 90, 13, 19, and 43; *V. mimicus* ATCC 33653, UM 4053, and UM 4074; *V. proteolyticus* ATCC 15338; *V. vulnificus* ATCC 27562 and UM 4304; *V. diazotrophicus* ATCC 33466; *V. alginolyticus* ATCC 17749 and UM 4109; *V. parahaemolyticus* ATCC 17802, ATCC 27969, UM 4111, UM 4324, UM 4108, and UM 4237; *V. damsela* ATCC 35083 and ATCC 33539; *V. carchariae* ATCC 35084; *V. anguillarum* ATCC 19264, UM 4393, and UM 4248; *V. aestuarianus* ATCC 35048; *V. cincinnatiensis* ATCC 35912; *V. natriegens* ATCC 14048 and UM 4384; *V. harveyi* ATCC 14126; *V. ordallii* ATCC 33509; *V. campbellii* ATCC 25920 and UM 4344; *V. fluvialis* NCTC 11328, UM 4476, and UM 4122; *V. tubiashii* 3358 and 3359; *V. splendidus* UM 4259 and UM 4424; *V. pelagius* UM 4422 and UM 4494; *V. furnissii* UM 4137; *Pseudomonas aeruginosa* ATCC 10145; *Pseudomonas putida* ATCC 12633; *Pseudomonas solanacerium* ATCC 11696; *Shigella sonnei* UM 773; *Shigella flexneri* ATCC 12022 and UM 774; *Serratia liquefaciens* NCTC 10442; *Serratia marcescens* ATCC 13880; *Proteus vulgaris* ATCC 13315 and UM 769; *Flavobacterium meningosepticum* ATCC 13253; *Photobacterium angustum* ATCC 25915; *Plesiomonas shigelloides* ATCC 14030; *Escherichia coli* H10407, ATCC 11303, and ATCC 11775; *Streptococcus faecalis* ATCC 19433; *Salmonella typhimurium* ATCC 14028; *Klebsiella pneumoniae* ATCC 13883; *Citrobacter freundii* ATCC 8090; *Enterobacter aerogenes* ATCC 13048; *Aeromonas caviae* ATCC 15468 and ATCC 15467; *Aeromonas media* ATCC 33907; *Aeromonas sobria* ATCC 9071; *Aeromonas hydrophila* UM 4396; and *Alcaligenes faecalis* ATCC 8750 (UM indicates the culture collection at University of Maryland).

RESULTS

Screening of bacterial strains for cross-reactivity with monoclonal antibody. All *Vibrio cholerae* O1 strains appeared as small distinct rods surrounded by a bright fluorescent green band. Fluorescent cells were not observed in preparations of the other 75 bacterial species tested.

Temperature and pH results. The temperatures of the river and pond water samples examined in this study ranged from 21 to 24°C. All pH readings were 6, and salinity values were 0.0‰. For tube wells, temperature readings ranged from 16 to 26°C, pHs ranged from 6 to 7, and salinities ranged from 0.4 to 0.5‰.

Enumeration of *V. cholerae* O1 in water. All Bangladesh village sites sampled were connected by the same river. In some villages, small ponds were sampled. Both river and pond water sources are used for bathing, washing, occasional drinking, and cooking. All villages had tube wells, installed to provide safe drinking water.

Enumeration data for water samples from village sites are shown in Table 1. AODC, done to enumerate total bacteria, revealed tube well counts of 10^4 to 10^6 cells per ml. River water samples contained 10^6 cells per ml, whereas pond water counts ranged from 10^6 to 10^7 cells per ml.

FA results for total *V. cholerae* O1 were negative for all tube well samples. The river water sample collected adjacent to the Kadamtali village contained 10^2 *V. cholerae* O1 cells per ml, whereas 10^3 cells per ml were present in all other river and pond water samples.

Results of viable *V. cholerae* O1 counts, enumerated by the FA-DVC procedure, yielded similar findings. As might have been predicted, *V. cholerae* O1 could not be detected by FA-DVC in tube well water samples examined in this study. Both pond and river water samples from Kadamtali yielded 10^2 viable *V. cholerae* O1 per ml. All other FA-DVC enumerations for pond and river water samples were about 10^3 cells per ml.

All most-probable-number estimates for water samples were <0.3 *V. cholerae* O1 cells per 100 ml, except for the river water sample collected at the village of Gajipur, which contained 2.0 cells per 100 ml (Table 1). This sample yielded the only culturable *V. cholerae* O1. No other isolates were recovered after screening of all yellow colonies growing on thiosulfate citrate-bile salts-sucrose agar culture plates by slide agglutination.

DISCUSSION

Critical to the success of the FA staining procedure described here was the specificity of the antibody used. Results of screening for cross-reactivity demonstrated ex-

TABLE 1. Enumeration of *Vibrio cholerae* O1 in Bangladesh waters by culture and FA methods

Sampling date	Site	Source	Cell count (cells/ml) by:			MPN[a] (100 ml)
			AODC	FA	FA-DVC	
1-8-86	Goalvaor	River	2.2×10^6	9.9×10^3	1.6×10^3	<0.3
1-8-86	Goalvaor	Tube well	2.5×10^6	0	0	<0.3
1-8-86	Shahwazkandi	Pond	2.7×10^7	7.7×10^3	5.9×10^3	<0.3
1-8-86	Shahwazkandi	Tube well	3.2×10^5	0	0	<0.3
1-8-86	Shahwazkandi	River	5.9×10^6	5.7×10^3	3.4×10^3	<0.3
1-8-86	Kadamtali	Tube well	8.5×10^4	0	0	<0.3
1-8-86	Kadamtali	Pond	1.9×10^7	2.9×10^3	9.4×10^2	<0.3
1-8-86	Kadamtali	River	6.8×10^6	4.1×10^2	1.6×10^2	<0.3
1-19-86	Sarderandi	Pond	4.4×10^6	2.9×10^3	1.5×10^3	<0.3
1-19-86	Sarderandi	River	2.9×10^6	5.4×10^3	1.9×10^3	<0.3
1-19-86	Uddamdi	Pond	2.0×10^7	6.1×10^3	1.4×10^3	<0.3
1-19-86	Uddamdi	River	4.9×10^6	3.5×10^3	1.8×10^3	<0.3
1-19-86	Gajipur	Pond	4.8×10^7	4.1×10^3	3.5×10^3	<0.3
1-19-86	Gajipur	River	1.9×10^6	4.4×10^3	1.6×10^3	2.0
1-19-86	Charmokundi	Pond	4.9×10^6	3.9×10^3	2.0×10^3	<0.3
1-19-86	Charmokundi	Pond 2	2.5×10^6	4.9×10^3	1.6×10^3	<0.3

[a] MPN, Most-probable-number estimate.

traordinarily high specificity of the monoclonal antibody for the *V. cholerae* O1 antigen. Because the reagent was to be employed in a microscopic procedure, a large number of bacterial strains were tested for cross-reactivity. By filtering a suspension of 10^6 to 10^7 cells per ml onto a filter and staining by FA, a large population of cells could be examined in 100 microscopic fields, and therefore any individual cells expressing antigens in common with the O1 antigen would be detected. No cross-reactivity was observed when representatives of the families *Vibrionaceae*, *Aeromonadaceae*, *Pseudomonadaceae*, *Enterobacteriaceae*, *Micrococcaceae*, and *Streptococcaceae* were tested. On the other hand, all *V. cholerae* O1 cells appeared bright and distinct when stained by FA.

The most-probable-number method was used to enumerate culturable *V. cholerae* O1. Sucrose-positive colonies were examined by slide agglutination, a tedious and time-consuming task requiring a minimum of 3 days from the time of sample collection to completion. *V. cholerae* O1 was not cultured from any sample except river water from Gajipur. Those isolates that were positive by slide agglutination were confirmed microscopically by FA staining with monoclonal antibody.

By fluorescence microscopic methods, enumeration of environmental samples was accomplished easily and more rapidly; e.g., a complete AODC required ca. 10 min per sample. These results served as a reference for determining what proportion of the total bacterial population in the water sample was made up of *V. cholerae* O1.

The FA procedure was used to enumerate the total *V. cholerae* O1 population. The FA-DVC variation enumerates viable cells. In this procedure, water samples were incubated with yeast extract and nalidixic acid. Substrate-responsive cells, i.e., viable cells, elongate because of inhibition of DNA gyrase (4, 8). After being stained with specific antibody and fluorescein conjugate, viable *V. cholerae* O1 cells appeared as long, fluorescent, green-banded bacilli when viewed under the microscope (1).

Approximately 3 h is required to complete the staining process for FA enumeration. An additional 6, 12, or 24 h is needed for the FA-DVC procedure, taking into account the requirement for supplemental incubation. The filter method used in these techniques permitted filtration of increasing volumes of water. The maximum volume filtered was dependent only on the turbidity of the water.

In all river and pond water samples examined in this study, total *V. cholerae* O1 cells constituted 0.01 to 0.1% of the total microbial population. Of the *V. cholerae* O1 population present, 16 to 85% was viable when examined by FA-DVC.

Although the tube well water samples yielded high AODC, results of FA analysis and FA-DVC indicated that *V. cholerae* O1 were not present. Tube wells have been installed in many villages to provide a source of safe drinking water. However, studies have shown that the rate of cholera infection is the same among inhabitants who drink water from tube wells as it is among those who do not. This finding was attributed to the multipurpose use of contaminated surface water, which was routinely used for bathing, cooking, and washing (9, 18, 19).

The International Centre for Diarrhoeal Disease Research, Bangladesh, conducts epidemiological examinations by conventional culture methods at those villages reporting cholera cases. Attempts to isolate *V. cholerae* O1 from food and water sources in the villages are not always successful (unpublished data). Our attempts to culture *V. cholerae* O1 were unsuccessful with all but one water sample, collected at the same time samples for FA analysis were collected. By the FA-DVC method, viable *V. cholerae* O1 could be detected and enumerated in all river and pond water samples examined. Viable *V. cholerae* O1 was detected in all samples that were culture positive as well as in samples that were culture negative.

It is apparent from these results that the FA procedure is a more sensitive detection method than standard culture procedures. The significant difference in results between the culture and FA procedures provides additional evidence for the existence of the viable but nonculturable stage of *V. cholerae* in the natural environment in an area in which cholera is endemic (1a).

Many environmental and laboratory studies have been done in which growth and survival of *V. cholerae* in water have been examined (2, 10, 16, 17, 20). Nutrient content, salinity, temperature, pH, and size of inoculum are some of the factors contributing to the persistence and recoverability of the organism. The survival time of *V. cholerae* in water may extend from hours to months. Viability in these experiments was determined by CFU appearing on plating media.

More-recent studies have shown that a variety of bacteria may enter a dormant state when physical and chemical conditions are less than optimal. The organisms remain viable, but nonculturable (1a, 13, 14; D. B. Roszak, Ph.D. thesis, University of Maryland, College Park, 1985). The FA-DVC technique has made it possible to detect, as well as to enumerate, specific viable cells that yield no growth by standard methods of cultivation.

It is not certain that all viable cells are capable of elongating under the outlined experimental conditions of the FA-DVC method. Yeast extract may be an inadequate substrate for bacteria in advanced stages of dormancy, as suggested by the results of Peele and Colwell (12). Furthermore, some bacteria may be resistant to the action of nalidixic acid. However, the FA-DVC method was significantly more successful at detecting viable *V. cholerae*, even though it may have underestimated the total viable count. Only viable cells are capable of elongation when exposed to the testing regime, and both laboratory and field data indicate that FA-DVC always exceed plate counts (1a, 13, 14; D. B. Roszak, Ph.D. thesis).

The role of zooplankton in the ecology of *V. cholerae*, i.e., their provision of a substrate for attachment and nutrient as well as a vehicle for transmission to humans, has been studied. Chitin, found on the surface of copepods, is capable of protecting microorganisms from the bactericidal action of gastric acids (6, 7, 11; D. R. Nalin, Letter, Lancet **ii**:958, 1976). That nonculturable bacteria can attach to plankton during unfavorable environmental conditions and, by ingestion, pass into the human gut, where optimal conditions for growth, replication, and pathogenic mechanisms exist, must be considered. Thus, viable but nonculturable bacteria cannot be ignored, especially since virulence of these bacteria as well as of injured bacteria has been demonstrated in animal studies (1a, 15).

In conclusion, FA-DVC provides a highly specific and sensitive detection system. Culturable as well as nonculturable bacteria can be observed and enumerated relatively quickly and easily. Village water sources in Bangladesh should be monitored by FA-DVC for viable *V. cholerae* O1, and the results should be correlated with the incidence of disease. When needed information becomes available, it will be possible to predict the risk of contracting cholera and describe the epidemiology of the disease.

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