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Effect of Transport at Ambient Temperature on Detection and Isolation of *Vibrio cholerae* from Environmental Samples

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It has long been assumed that prolonged holding of environmental samples at the ambient air temperature prior to bacteriological analysis is detrimental to isolation and detection of *Vibrio cholerae*, the causative agent of pandemic cholera. The present study was aimed at understanding the effect of transporting environmental samples at the ambient air temperature on isolation and enumeration of *V. cholerae*. For water and plankton samples held at ambient temperatures ranging from 31°C to 35°C for 20 h, the total counts did not increase significantly but the number of culturable *V. cholerae* increased significantly compared to samples processed within 1 h of collection, as measured by culture, acridine orange direct count, direct fluorescent-antibody–direct viable count (DFA-DVC), and multiplex PCR analyses. For total coliform counts, total bacterial counts, and DFA-DVC counts, the numbers did not increase significantly, but the culturable plate counts for *V. cholerae* increased significantly after samples were held at the ambient temperature during transport to the laboratory for analysis. An increase in the recovery of *V. cholerae* O1 and improved detection of *V. cholerae* O1 *rfb* and *ctxA* also occurred when samples were enriched after they were kept for 20 h at the ambient temperature during transport. Improved detection and isolation of toxigenic *V. cholerae* from freshwater ecosystems can be achieved by holding samples at the ambient temperature, an observation that has significant implications for tracking this pathogen in diverse aquatic environments.

Cholera is an highly epidemic diarrheal disease which continues to devastate populations of many developing countries in which socio-economic conditions are poor, sanitary systems and public hygiene are rudimentary, and safe drinking water is limited. The conventional microbiological methods for enumerating and isolating *Vibrio cholerae* involve cultural, biochemical, and immunological assays (10). Several investigators have developed PCR and DNA probe-based techniques for direct detection of pathogenic *Vibrio* species (2, 3, 8). A variety of other techniques for direct detection of *V. cholerae* in clinical and environmental samples, including chromatographic assays (28), direct fluorescent-monoclonal-antibody methods (1, 11), and monoclonal antibody-based kits (4), have also been described. The laboratory protocols for isolation of *V. cholerae* from clinical specimens from cholera patients include both direct plating and broth enrichment methods (20). In the case of environmental samples, despite the many methods that have been devised, overnight enrichment in alkaline peptone broth with isolation on thiosulfate-citrate-bile salts-sucrose (TCBS) medium immediately after collection is most frequently employed (5, 23).

In Bangladesh, cholera epidemics are recurrent. However, the lack of laboratory facilities in remote areas of the country require samples to be transported from the site of collection to a central laboratory before processing, and road links are poor. In most cases, the transport time between sample collection and processing is relatively long, ranging from hours to a day. The effect of such lengthy transportation times for environmental samples held at the ambient air temperature on the microbial population of samples, particularly the effect on isolation and detection of *V. cholerae*, was investigated in this study. We report here not only that there was an increase in the overall microbial load, a phenomenon that has been recognized for a long time, but also that we had significantly greater success in isolating and identifying toxigenic *V. cholerae* O1, a notoriously difficult task in the case of environmental samples. The presence of chitinous exoskeletons of plankton and the high ambient air temperatures (31 to 35°C) in Dhaka, Bangladesh, are considered the most probable factors that contributed to the increased *Vibrio* viable count.

MATERIALS AND METHODS

Sample collection and processing. Water and plankton samples from Dhanmondi Lake, a freshwater ecosystem, were collected weekly during April and May 2004, when the average air temperatures ranged from 31°C to 35°C. Samples were collected aseptically in sterile glass bottles and transported at the ambient temperature from the site of collection to the laboratory for analysis. For comparison, as described elsewhere (7), samples were processed immediately after collection and again after incubation at the ambient temperature for 20 h during transportation.

For sample collection, 100 liters of water was filtered successively through 64-μm and 20-μm mesh nylon nets (the 64-μm net was placed on the 20-μm net) (Millipore Corp., Bedford, MA), and 50-ml portions of the concentrates from the collecting buckets (at the base of each net) were collected initially to obtain crude measurements for the zooplankton and phytoplankton, respectively. Also, during filtration, the filtrate water from the 20-μm mesh net was collected as a

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TABLE 1. PCR primers used in this study

Primer		Sequence	Size of amplicon (bp)	Reference
No.	Designation			
1	*ctx*AF	5′-CTCAGACGGGATTTGTTAGGCACG-3′	302	14
2	*ctx*AR	5′-TCTATCTCTGTAGCCCCTATTACG-3′	302	14
3	O1 *rfb*F	5′-GTTTCACTGAACAGATGGG-3′	192	14
4	O1 *rfb*R	5′-GGTCATCTGTAAGTACAAC-3′	192	14
5	O139 *rfb*F	5′-AGCCTCTTTATTACGGGTGG-3′	449	14
6	O139 *rfb*R	5′-GTCAAACCCGATCGTAAAGG-3′	449	14
7	*ompW*F	5′-CACCAAGAAGGTGACTTTATTGTG-3′	304	26
8	*ompW*R	5′-GGTTTGTCGAATTAGCTTCACC-3′	304	26

representative water sample and was used to determine the presence of planktonic (free-living) bacterial populations.

Both zooplankton and phytoplankton samples were concentrated further in the laboratory using plankton nets (in specially designed plastic beakers) to obtain a final volume of 5 ml and were homogenized using a glass homogenizer (Elberbach Corp., Ann Arbor, Mich.) prior to bacteriological analyses. The homogenates were used for direct plating, as well as for enrichment for *V. cholerae*. The bacterial counts for water samples were obtained with water concentrated by passage through a 0.22-μm bacteriological membrane (Millipore Corp., Bedford, MA). All samples were subjected to direct plating, enrichment, heterotrophic plate count, total *Vibrio* count, total *V. cholerae* count, direct fluorescent-antibody–direct viable count (DFA-DVC) (11), and acridine orange direct count (AODC) analyses (12).

Heterotrophic plate count, coliform count, and total *Vibrio* count analyses. One hundred microliters of each of the samples was plated on Luria-Bertani agar, fecal coliform (mFC) agar, and TCBS agar (Difco Laboratories, Detroit, Mich.) for heterotrophic plate count, fecal coliform count, and total *Vibrio* count analyses, respectively. Luria-Bertani agar was used for heterotrophic plate count analyses because this nonselective medium provides better support for stressed cells (16) and produces discrete (larger) colonies overnight (M. Alam, A. Sadique, N. A. Hasan, N. A. Bhuiyan, G. B. Nair, A. K. Siddique, D. A. Sack, R. B. Sack, A. Ali, A. Huq, and R. R. Cowell, unpublished data). For heterotrophic plate count analyses, samples were subjected to 10-fold dilution prior to spread plating. Luria-Bertani and TCBS agar plates were incubated at 37°C, and the mFC agar was incubated at 44.5°C for 18 to 24 h. Yellow colonies on TCBS agar were considered total *Vibrio* colonies and counted, and they were subcultured onto gelatin agar (Difco). Gelatinase-positive colonies were spread plated on gelatin agar and incubated at 37°C for 18 to 24 h after addition of disks (Millipore Corp., Bedford, MA) containing 150 μg of the vibriostatic compound pteridine (Sigma). Colonies of the pure culture were picked and tested for catalase and oxidase activities. Catalase- and oxidase-positive colonies were subjected to PCR for amplification of the *V. cholerae* species-specific 304-bp *ompW* gene (26).

AODC analysis. The AODC analysis was carried out by using the method described elsewhere (12). Briefly, 25-mm polycarbonate Nuclepore membranes were prestained by soaking them for 2 to 24 h in irgalan black (2%) and acetic acid. Approximately 1 to 2 ml of each sample (plankton or water) was filtered through a 0.2-μm prestained Nuclepore membrane (Millipore Corp., Bedford, MA). Counterstaining was performed using acridine orange (final concentration, 0.1%; OmniPur; EM Science, Gibbstown, NJ) for total cell counting. Filters were mounted on microscope slides by using low-fluorescence immersion oil (type A or FF) and were examined by using a ×100 oil immersion lens and an epifluorescence microscope (BX51; Olympus, Japan) equipped with a 420-nm UV filter. Ten microscope fields were counted by using the method described elsewhere (24). The cell concentration was computed as follows: number of cells per milliliter = (average number of cells per square) × (number squares per filter) × (dilution factor)/sample volume.

Enrichment and plating. Samples were enriched by incubation in alkaline peptone water (APW) (Difco) at 37°C for 6 to 8 h before plating, using methods described previously (23), and then 5 μl of enriched APW broth was streaked onto TCBS agar and taurocholate-tellurite-gelatin agar and incubated at 37°C for 18 to 24 h. The identities of suspected *Vibrio*-like colonies were confirmed using selected biochemical tests, PCR for the *V. cholerae*-specific *ompW* gene (26), and serological tests employing polyvalent and monoclonal antibodies specific for *V. cholerae* O1 or O139.

DFA-DVC analysis. The DFA-DVC analysis was carried out by using the procedure described previously (11). Briefly, samples were incubated in the dark at room temperature for 6 to 8 h in the presence of 0.025% yeast extract and 0.002% nalidixic acid. Under these conditions, the viable bacterial cells elongate from the coccoid nonculturable form to rod-like cells (22). This is due to the inhibitory effect of nalidixic acid (a DNA gyrase inhibitor), which prevents the *V. cholerae* cells from multiplying. Samples were then centrifuged, and the pellet was resuspended in 1 ml of phosphate-buffered saline and processed by following the instructions of a DFA O1 kit (11); 10-μl portions of samples were air dried on the groove of a specialized microscope slide, fixed with ethanol, and labeled for 30 min (in the dark) with a *V. cholerae* DFA reagent specific for *V. cholerae* O1 (Cholera DFA; New Horizons Diagnostics Corporation, Columbia, MD). The slide with the antigen-antibody mixture was washed with phosphate-buffered saline to get rid of the free or nonspecifically bound antibody, air dried (in the dark), treated with mounting fluid, and examined at a magnification of ×1,000 under the epifluorescence microscope (Olympus model BX51) equipped with an HBO 200W/2 mercury lamp (OSRAM, Munich, Germany) and a digital camera (Olympus model DP20) connected to a computer (Dell). The fluorescent green cells were counted by using the method described elsewhere (24).

PCR detection of *V. cholerae* O1 *rfb*, *ctx*, and *ompW* genes. The *ctxA* and *rfb* genes of *V. cholerae* O1 were amplified by multiplex PCR by using methods described elsewhere (14), whereas the *ompW* gene was amplified by PCR with a single pair of primers (20). For PCR amplification, samples were enriched by incubation in APW (Difco) at 37°C for 6 to 8 h and then preincubated with 0.025% yeast extract (Difco) and 0.002% nalidixic acid, using the method described previously (21). Briefly, the broth culture was centrifuged at ca. 10,000 rpm to collect cell pellets. DNA was extracted from the pelleted cells and subjected to PCR. The primer sequences for the genes (e.g., *rfb* for both *V. cholerae* O1 and O139 and *ctxA*) are listed in Table 1. Amplification with the three primer pairs was performed simultaneously in 0.2-ml microcentrifuge tubes. Samples (3 μl) were added to the PCR mixture (final volume, 30 μl) containing each deoxynucleoside triphosphate at a concentration of 0.21 mM, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), each member of the *ctxA* primer pair at a concentration of 0.27 μM, and 0.75 U of *Taq* polymerase (Takara, Kyoto, Japan). The amplification conditions used were 5 min at 94°C for initial denaturation of the DNA, 35 cycles consisting of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, and a final extension step for 7 min at 72°C in a DNA Robo Cycler gradient temperature cycler (Stratagene, La Jolla, CA). After amplification, 6 μl of each reaction mixture was separated by electrophoresis on a 3% agarose gel, and the amplified gene products were visualized under UV light after staining with ethidium bromide.

All experiments were carried out during the dry summer season in Dhaka, Bangladesh, as indicated above. Because the study was designed to determine the impact of prolonged transport at the ambient temperature on the isolation and detection of *V. cholerae* from environmental samples (water, >64-μm plankton fraction, and 20- to 64-μm plankton fraction), three samples of each type were collected in each round from a freshwater lake (Dhanmondi, Dhaka), and the experiment was repeated at least three times to ensure reproducibility of the results. The statistical analyses of data were performed by using a *t* test, and *P* values were determined. A difference was considered significant if the *P* value was <0.05.

RESULTS AND DISCUSSION

The heterotrophic plate counts for total aerobic bacteria in water, 20-μm plankton fraction, and 64-μm plankton fraction samples processed immediately after collection increased by less than 1 log (Table 2); however, the differences were not

TABLE 2. Comparative counts of total microbial populations and fecal coliforms in environmental samples processed immediately after collection and processed again after 20 h of incubation at the ambient temperature[a]

Sample[b]	Log_{10} bacteria/ml (mean ± SEM)					
	Heterotrophic plate counts		AODC		Fecal coliforms	
	Immediate processing	Delayed processing	Immediate processing	Delayed processing	Immediate processing	Delayed processing
Water	3.68 ± 0.52	3.74 ± 0.34	4.47 ± 0.06	4.67 ± 0.27	1.72 ± 0.63	1.69 ± 0.81
>64-μm plankton fraction	1.70 ± 0.56	2.48 ± 1.18	1.91 ± 0.11	2.55 ± 0.19		
20- to 64-μm plankton fraction	1.93 ± 0.21	2.88 ± 0.67	1.89 ± 0.09	2.01 ± 0.17		

[a] Heterotrophic plate counts for total culturable aerobic bacteria (CFU/ml) and AODC for culturable and viable but nonculturable bacteria (cells/ml) in water, the 20- to 64-μm plankton fraction, and the >64-μm plankton fraction were determined. Fecal coliforms (cells/ml) were also counted in unfiltered water.
[b] Samples ($n = 3$) were collected weekly from a freshwater ecosystem and subjected to bacteriological analysis.

significant statistically when the samples were processed after incubation at the ambient temperature for 20 h. Also, no changes in fecal coliform counts were observed after transport at the ambient temperature (Table 2), in accordance with results reported by other investigators (13). Fecal coliform bacteria serve as an indicator of pollution from domestic waste, and a consistent characteristic of this indicator organism is its presumed low level of multiplication under environmental conditions (27), as was the case in this study (no significant change in the fecal coliform counts was observed after transport under the ambient temperature conditions).

The total culturable *Vibrio* counts were below detectable levels in both the water and 20-μm plankton fraction samples as determined by all methods used (data not shown). However, the 64-μm plankton fraction samples, in which there was no growth of *V. cholerae* when samples were processed immediately after collection, had mean *Vibrio* counts of 700 CFU/ml when the samples were processed after incubation for 20 h at the ambient temperature during transport. The *Vibrio* counts comprised the total culturable *Vibrio cholerae* counts, as did the counts for sucrose-fermenting, oxidase-positive colonies on TCBS agar that were also positive for the 304-bp *ompW* gene specific for *V. cholerae* (data not shown). These results indicate that there was selective enrichment for *V. cholerae* during 20 h of incubation at the ambient temperature for the 64-μm plankton fraction (zooplankton) samples. *V. cholerae*, including *V. cholerae* O1, is a chitin-digesting organism (6), and the zooplankton (predominantly copepods) comprising the zooplankton populations in Bangladesh lakes and ponds (15) have chitinous exoskeletons. Early studies of *Vibrio parahaemolyticus* and chitin enrichment by Kaneko and Colwell (17) showed that *V. parahaemolyticus* both bound to chitin and comprised the commensal flora of zooplankton in Chesapeake Bay. There appears to be a similar phenomenon for *V. cholerae* in the Bay of Bengal, based on the results of this study.

We interpret the results presented here to indicate that despite a significant increase in the *V. cholerae* counts, the total culturable bacterial counts did not increase significantly after the environmental samples were held for 20 h at the ambient temperature during transport, as determined by the aerobic heterotrophic plate count, total culturable *Vibrio* count, and total culturable *V. cholerae* count analyses. The total direct counts obtained by AODC analysis also remained approximately the same (Table 2); i.e., there was not a statistically significant change in the direct counts determined by the AODC analysis, despite the increase in *Vibrio* growth in the culture after it was held at the ambient temperature. This interesting result was attributed to selective enrichment of viable but nonculturable *V. cholerae* populations that did not appear on culture plates until after the increase in the ambient temperature and enrichment in the presence of the concentrated 64-μm plankton fraction. Since the *V. cholerae* count analysis was negative for samples processed immediately after collection and high counts were obtained for total *Vibrio* spp., especially *V. cholerae*, after samples were held at 31 to 35°C in the presence of the concentrated 64-μm plankton during transport, this finding suggests that the potential "trigger" for cholera epidemics is the seasonal zooplankton bloom that occurs with elevated surface water temperatures. The association of *V. cholerae* with zooplankton, which has been shown to be a reservoir for *Vibrio* spp., including *V. cholerae* (5, 17), is reflected in the dramatic recovery of *V. cholerae* from samples enriched in the presence of zooplankton (that is, the 64-μm plankton fraction that was used to obtain a crude estimate of zooplankton). An overall increase in the concentration of several microbiological species, including *Vibrio* spp., has been reported to be related to the duration and temperature of storage of shellfish (13). Notably, incubation at elevated temperatures (17°C and 22°C) was reported to result in large increases in the total counts of *Vibrio vulnificus* in shucked oysters (25). The results of the study reported here provide the first documentation of increased recovery of *V. cholerae* O1

TABLE 3. Comparative recovery of *V. cholerae* serovar O1 and non-O1/O139 from water, the >64-μm plankton fraction, and the 20- to 64-μm plankton fraction enriched immediately after collection or enriched after incubation at the ambient temperature for 20 h during transport

Sample[a]	No. of *V. cholerae* colonies confirmed (mean ± SEM)			
	Serovar O1		non-O1/non-O139	
	Immediate processing	Delayed processing	Immediate processing	Delayed processing
Water	7.67 ± 4.04	12.33 ± 1.53	11.33 ± 1.53	12.67 ± 4.04
>64-μm plankton fraction	2.67 ± 0.58	23.00 ± 2.00	6.00 ± 1.50	27.67 ± 2.52
20- to 64-μm plankton fraction				6.00 ± 1.00

[a] Samples ($n = 3$) were enriched in APW at 37°C for 6 to 8 h, and 0.1 ml of the enriched APW was diluted 10-fold before spread plating on TCBS agar and taurocholate-tellurite-gelatin agar, as described elsewhere (23).

TABLE 4. Comparative DFA-DVC for culturable and viable but nonculturable *V. cholerae* in environmental samples processed immediately after collection and again after 20 h of incubation at the ambient temperature

Sample[a]	Log_{10} *V. cholerae* cells/liter (mean ± SEM)	
	Immediate processing	Delayed processing
Water	5.61 ± 0.96	6.19 ± 0.52
>64-μm plankton fraction	1.27 ± 0.40	1.64 ± 0.50
20- to 64-μm plankton fraction	2.90 ± 1.34	3.36 ± 1.05

[a] Samples ($n = 3$) were incubated in the dark at room temperature for 6 to 8 h in the presence of 0.025% yeast extract and 0.002% nalidixic acid, treated with antibody, and examined at a magnification of ×1,000 under the epifluorescence microscope (Olympus model BX51) and by following the instructions of a DFA O1 kit (11).

from environmental samples enriched with concentrated zooplankton and incubated at 31 to 35°C for ca. 20 h.

The enrichment of environmental samples in alkaline peptone water with zooplankton concentrated from the same samples resulted in significant increases in recovery of both *V. cholerae* serogroup O1 and non-O1 *V. cholerae* when the samples were processed after 20 h of holding at the ambient temperature (Table 3). Although a significant change in the recovery was not observed for water containing planktonic (free-swimming) *V. cholerae*, for the zooplankton samples there was a significant increase in the recovery of *V. cholerae* O1 when the samples were enriched after preincubation for 20 h at the ambient temperature. There was also a significant increase in the recovery of non-O1 and non-O139 *V. cholerae* for phytoplankton and zooplankton samples enriched and incubated at the ambient temperature for 20 h, whereas no growth of *V. cholerae* was recorded for phytoplankton samples that had been enriched and processed like the zooplankton samples immediately after collection (Table 3). We concluded that the increased recovery of toxigenic *V. cholerae* O1 from environmental samples after preenrichment holding of samples for 20 h at the ambient air temperature occurred because the concentrated zooplankton samples selectively enriched for *V. cholerae*. Other investigators have reported enhanced growth of *V. vulnificus* during incubation at the ambient temperature (7, 19, 25) but without specific nutrient enrichment. Interestingly, delayed secondary enrichment has been proposed for isolation of *Salmonella* from poultry and poultry environments (29). Higher rates of recovery of *V. cholerae* from oysters and greater specificity, as determined by the confirmation rate for suspect colonies, have also been reported (9, 18). The results of such studies provide support for the conclusion drawn from the results obtained here, which collectively rule out the currently accepted notion that prolonged storage of samples at the ambient temperature following collection is detrimental to *V. cholerae*.

In contrast to the results of direct plating of the environmental samples on agar media, which reflect only the culturable population, the DFA-DVC results reflect both culturable and viable but nonculturable cells of *V. cholerae* (1). Using the DFA-DVC method, the counts of *V. cholerae* O1 in water, phytoplankton, and zooplankton samples did not increase to a

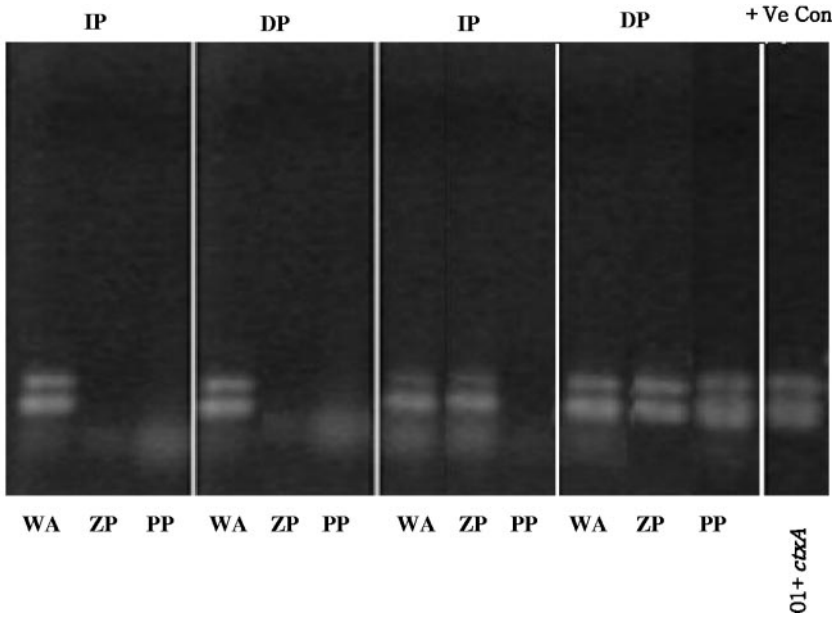

FIG. 1. Direct detection of *V. cholerae* O1 *rfb* and *ctxA* after immediate processing (IP) or delayed processing (DP) of samples of water (WA), the 64-μm plankton fraction (ZP), and the 20-μm plankton fraction (PP) by multiplex PCR. The first two gels from the left show the PCR results for sampling round 1, in which amplification of *V. cholerae* O1 *rfb* and *ctxA* occurred after immediate processing in the water sample and remained unchanged after delayed processing even after 20 h of preincubation at the ambient temperature. The third and fourth gels from the left show the PCR results for sampling round 2 after immediate processing and delayed processing, respectively. The amplification of the *V. cholerae* O1 *rfb* and *ctxA* genes that occurred in water and zooplankton samples but not in phytoplankton (20-μm fraction) samples after immediate processing, however, occurred in samples subjected to delayed processing (i.e., after 20 h of incubation at the ambient temperature). +Ve Con, positive control for *V. cholerae* O1 *rfb* and *ctxA*.

level of significance when the samples were processed after 20 h of holding at the ambient temperature (Table 4). The lack of a significant increase in the DFA-DVC value for *V. cholerae* O1 despite stimulated counting and recovery of *V. cholerae* after delayed processing, however, supports the hypothesis that the selective enrichment of viable but nonculturable *V. cholerae* may be attributed to the effect of 20 h of holding at the ambient temperature during transport from the field to the laboratory. *V. cholerae* O139 was, however, not detected in the environmental samples examined in the present study.

Multiplex PCR for the detection of *ctxA* and *rfb* genes for O1 and O139 revealed amplification of both *ctxA* and O1 *rfb* genes in the water and zooplankton samples processed immediately and after holding at the ambient temperature. The *ctxA* and O1 *rfb* genes, which were not detected in the phytoplankton (20-μm fraction) samples processed immediately, however, were positive for samples subjected to delayed processing, i.e., after 20 h of incubation at the ambient temperature (Fig. 1). We concluded that the improved detection of the O1 *rfb* and *ctxA* genes was the result of selective enrichment of toxigenic *V. cholerae* O1 and was attributable to providing primers with more templates. Also, work in progress has shown that the 20-μm plankton fraction contains copepod eggs and nauplii (unpublished data).

Finally, the overall findings of the present study can be interpreted to indicate that holding samples isolated from a freshwater ecosystem at ambient temperatures ranging from 31°C to 35°C for 20 h did not have deleterious effects but rather facilitated the growth of the total heterotrophic bacteria, a result that was not unexpected based on the results of many previous microbial ecology studies. However, the concomitant selective increases in the total culturable *Vibrio* populations, particularly the *V. cholerae* population, were unexpected. We concluded that isolation of *V. cholerae* both by culture and by detection and estimation using DFA-DVC and multiplex PCR after environmental samples are held at the ambient temperature or an elevated temperature prior to processing is a useful method for enhancement of isolation of *V. cholerae* from environmental samples. Clearly, to determine the total number of heterotrophic bacteria in environmental samples, an AODC analysis done immediately after collection is recommended. On the other hand, counting of *V. cholerae* cells after prolonged incubation at the ambient temperature during transport of environmental samples from the field to the laboratory, as shown here, provided improved isolation and detection of toxigenic *V. cholerae* from freshwater ecosystem, a finding that may also have significant implications for monitoring this human pathogen in diverse aquatic environments.

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