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# Critical Factors Influencing the Occurrence of *Vibrio cholerae* in the Environment of Bangladesh

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**The occurrence of outbreaks of cholera in Africa in 1970 and in Latin America in 1991, mainly in coastal communities, and the appearance of the new serotype *Vibrio cholerae* O139 in India and subsequently in Bangladesh have stimulated efforts to understand environmental factors influencing the growth and geographic distribution of epidemic *Vibrio cholerae* serotypes. Because of the severity of recent epidemics, cholera is now being considered by some infectious disease investigators as a "reemerging" disease, prompting new work on the ecology of vibrios. Epidemiological and ecological surveillance for cholera has been under way in four rural, geographically separated locations in Bangladesh for the past 4 years, during which both clinical and environmental samples were collected at biweekly intervals. The clinical epidemiology portion of the research has been published (Sack et al., J. Infect. Dis. 187:96–101, 2003). The results of environmental sampling and analysis of the environmental and clinical data have revealed significant correlations of water temperature, water depth, rainfall, conductivity, and copepod counts with the occurrence of cholera toxin-producing bacteria (presumably *V. cholerae*). The lag periods between increases or decreases in units of factors, such as temperature and salinity, and occurrence of cholera correlate with biological parameters, e.g., plankton population blooms. The new information on the ecology of *V. cholerae* is proving useful in developing environmental models for the prediction of cholera epidemics.**

Cholera has historically occurred in periodic epidemics, with the most severe epidemics limited to a few countries, namely Bangladesh, India, and countries in Africa and South America. During the past three decades, however, this disease has occurred in geographical areas from which it had seemingly disappeared almost a century ago (35). Including these new appearances, epidemics have been reported in over 75 countries in South America, Africa, and Asia during the past decade (38). In addition, each year sporadic cases are reported in other countries around the world (38).

Interestingly, cholera is one of the few bacterial diseases known for its pandemicity, and until 1992, all epidemics of cholera were caused by *Vibrio cholerae* serogroup O1. In the latter part of 1992, a newly recognized O139 serogroup was isolated in areas surrounding the Bay of Bengal and was linked to major epidemics, first in Madras on the eastern coast of India and then in the southern part of Bangladesh. Later it was detected in neighboring countries and has continued to persist in that geographic region (6, 28).

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In 1992 in Bangladesh during a 12-week period, there were approximately 220,000 cases of cholera caused by serotype O139, with over 8,000 deaths, more deaths than in all of Latin America that same year (31, 35). Cholera is known to be a disease with a high mortality (≈60% if untreated); with adequate treatment (intravenous and oral rehydration therapy, supplemented with appropriate antibiotics) the mortality drops to <1.0% (5, 26). The large numbers of deaths indicate that adequate therapy was not available to the many persons who died (6, 35).

In the recent history of cholera, most major epidemics originated in coastal regions, including both the South American epidemic that began in the coastal regions of Peru, spreading to 21 countries, including Mexico, and the new O139 outbreak in India and Bangladesh. In Dhaka City and a rural area of Bangladesh, Matlab, cholera occurs year-round, with a distinct pattern of two peaks of disease, one in the spring and the other in the fall (16, 30).

The presence of *V. cholerae* O1 year-round via its commensal association with plankton was established by Colwell and coworkers using direct detection methods (17). It is still not certain what triggers the continuing seasonal epidemics of cholera in Bangladesh and what determines the persistence and multiplication of *V. cholerae* O1 and O139 in the cholera-endemic regions of the world. However, coexistence of *V. cholerae* O1 and O139 serogroups in association with plankton has

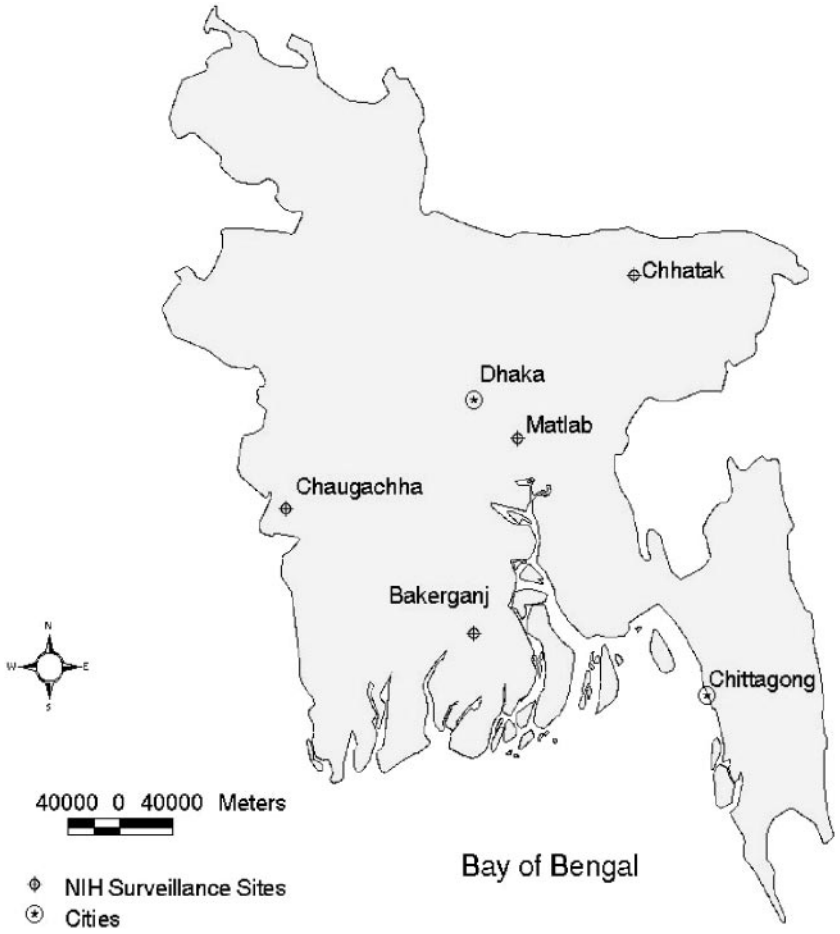


FIG. 1. Four sites, Bakerganj, Chaugachha, Chhatak, and Matlab, where samples were collected every 2 weeks between March 1997 and December 2000. (Reprinted from reference 29 with permission of the publisher.)

been documented in Bangladesh (18). Furthermore, failure to culture *V. cholerae* even in the cholera-endemic region throughout the year, when they can be observed using direct detection methods, raises the question of whether these bacteria persist in a state escaping culture (17).

In laboratory microcosm experiments, normal healthy cells of *V. cholerae* O1 were induced to become nonculturable, and these bacteria were shown to produce fluid in rabbit ileal loops (7) and to cause clinical symptoms of cholera in humans (10). Although the ability of nonculturable *V. cholerae* O1 to produce disease is not always successful (33), it is unsafe to deny the presence of these viable bacterial cells, as has been suggested by a few other investigators (4).

Although the autochthonous existence of *V. cholerae* in the aquatic environment of cholera-endemic regions has been established (8, 14, 21), the triggering factor or factors initiating cholera epidemics in one or several different geographical locations at approximately the same time remains elusive. Cholera is well recognized as a "water-borne" disease, with close linkage of the population dynamics of the causative agent with selected physical, chemical, and biological parameters of natural waters (11, 21, 22). Climatological factors have recently been shown also to be significant in cholera epidemics (9, 19). Notably, it has been established by remote sensing, employing satellites, that sea surface temperature and sea surface height are correlated with cholera epidemics (9, 24). Studies in Lima, Peru, have also suggested that increasing water temperature can serve as a trigger for the occurrence of epidemic disease (13, 14).

The objective of this study was to elucidate the influence of specific environmental factors on outbreaks of cholera, with the ultimate goal being to develop a model for predicting cholera to allow intervention in and/or prevention of cholera epidemics.

## MATERIALS AND METHODS

The study reported here was conducted from March 1997 to December 2000. Bimonthly sampling in four widely separated areas of rural Bangladesh was accomplished to determine the physical, chemical, and biological parameters of the natural bodies of water used by villagers as a source of drinking and household water.

**Surveillance sites.** Four locations were selected for surveillance, representing different geographical locales of Bangladesh (Fig. 1). Each sampling location included four sites, e.g., rivers, ponds, and lakes. Sampling sites were carefully selected, with attention to terrain, human population encompassed, and availability of hospital service for the individuals participating in the study.

**Description of the Sampling Sites. (i) Bakerganj.** Bakerganj is located at the upper edge of the southern estuary of Bangladesh, a cholera-prone area in the district of Barisal, and situated at the southwestern part of Bangladesh, near the Bay of Bengal. One river, one pond, and two lakes were included in the sampling

protocol. A major river, Tulatali, flows through Bakerganj and the Thana Health Complex, a government-run community-level rural health facility with hospital beds, located approximately 2 km west of Bakerganj. The Thana Health Complex is also an administrative unit, with a police station, and is composed of groups of villages. Several man-made lakes are located in this area, one of which is situated in a village, Rohitarpur, and is heavily used by the nearby villagers as well as children from a primary school and users of a mosque. The ponds in these villages are generally used for domestic purposes and were also included in the study.

**(ii) Chhatak.** Chhatak is located in the Sunamganj district, 40 km from the divisional town of Sylhet. Among the four study sites included in this district are a river and three ponds but no lake. One of the ponds is adjacent to the Chhatak Thana Health Complex. The Surma River flows through the district and is heavily polluted with industrial wastes from an adjacent pulp mill and a cement factory. During monsoon flooding, the Surma River overflows its banks. Also, there are several blacksmithies located on the western bank of one of the ponds. Other ponds are situated in the district, and zooplankton and phytoplankton blooms occur regularly in these ponds.

**(iii) Chaugachha.** Chaugachha is also a thana (political jurisdiction with a police station) in the Jessore district. The distance from Chaugachha to Dhaka is ca. 300 km southwest. Among the four sampling sites in Chaugachha are two ponds, a river, and a lake. All the water bodies and the amount of water used daily by the villagers were similar here and elsewhere in rural Bangladesh. The major river flowing through the thana is the Kapotakkho, once known as a mighty river of Bangladesh. The sampling area is located next to the Chaugachha Bazaar, to which people travel daily or weekly from significant distances to shop and trade. One of the lakes is a natural lake used for aquaculture and is administered by the Department of Fisheries, Government of Bangladesh. This lake has no inlet or outlet but receives runoff from adjacent agricultural land. The study pond is located near a mosque and a religious school, and hence it has a large number of daily users.

**(iv) Matlab.** Matlab Thana is in the district of Chandpur and is a well-known riverine area located in central Bangladesh. Cholera is highly endemic here, and the area has been under cholera and demographic surveillance by the International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B) since the early 1960s. Located in the delta formed by two major rivers, the Meghna and the Ganges, Matlab is 55 km southeast of the capital city of Dhaka. The major river that passes through Matlab is the Dhonagada, a branch of the Meghna. During monsoon flooding, the banks of the river are submerged. The sampling site is situated in the village of Kadamtali, near the Matlab bazaar, 200 m from the mouth of a canal which flows along the ICDDR,B Hospital in Matlab. Previously part of a canal, this body of water became a lake after establishment of an embankment 30 years ago and was included in the study. One of the study ponds in Matlab covers approximately 1 acre and is very large by Bangladesh village standards and is located in the village of Dagarpur. As noted above, it is common for a mosque to be located near a pond, with the pond providing a source of water for those who frequent the mosque. The other pond included in the study is located outside the embankment and is subject to regular flooding and overflowing of its banks.

In selecting sites for sample collection, attention was given to whether neighboring villagers also used the water, as well as to the location of farmhouses, since leaching from cowsheds and open latrines is, unfortunately, common to these water bodies. The Thana Health Complex was an important factor in selecting the sampling sites in Matlab, because it is recognized as the treatment hospital for diarrheal diseases and maintains records on cholera. Matlab includes the International Centre for Diarrheal Disease Research, Bangladesh, Dhaka field hospital, which has a diarrheal surveillance record covering more than 35 years. Each site included catchments for an estimated population of 140,000 to 200,000 villagers.

Details of the clinical surveillance have been published elsewhere (29). In brief, a physician examined patients at each of the surveillance hospital centers during a 3-day period every 2 weeks, and complete clinical and microbiological investigations were done to determine the cause of the diarrheal cases that arrived at the hospitals.

Environmental monitoring and clinical surveillance for cholera were conducted at 15-day intervals, beginning in March 1997 at Matlab and Chhatak and in June 1997 at Bakerganj and Chaugacha. Monitoring of all sites continued until December 2000.

**Environmental analysis. (i) Sample collection.** Water, zooplankton, phytoplankton, sediment, and floating vegetation (*Eichhornia crassipes*, *Pistia* spp., and *Lemna* spp.) samples were collected every 2 weeks at each site. One liter of water was collected in presterilized narrow-mouth plastic bottles. Plant samples were collected using sterile plastic bags and sterile tongs. Sediment samples were collected using a core sampler (constructed at ICDDR,B) and transported to the laboratory in 125-ml sterile glass bottles. Plankton samples were collected by filtering 100 liters of water through a plankton net (mesh size 64 μm for zooplankton >64 μm in size and 20-μm net for phytoplankton >20 μm in size) previously disinfected with 70% ethyl alcohol. Plankton samples were concentrated to 50 ml and stored in sterile glass bottles. All samples were transported to the laboratory in an insulated box to maintain a temperature close to that of the water at each collection site and were processed within 24 h of collection.

Phytoplankton characterization was done using 1-liter water samples collected in narrow-neck (Nalgene, Fisher Scientific) plastic bottles and amended with 3 ml Lugol's iodine solution (final concentration 0.3%). Bottles were incubated in the dark without shaking at room temperature for 48 to 72 h to sediment the plankton. Approximately 900 ml of supernatant was carefully decanted and 100 ml of the concentrated plankton from the bottom of the bottle was transferred to a 125-ml bottle and preserved by addition of formaldehyde to a final concentration of 4%, vol/vol.

The 20-μm phytoplankton samples, after fixation with formaldehyde, were enumerated using a Sedgewick-Rafter counting cell (2). Both phytoplankton and zooplankton were enumerated, characterized, and identified following published methods (32, 37).

**(ii) Physicochemical parameters.** Air and water temperature, water conductivity, and salinity were measured using a portable meter (HACH model CO150 conductivity meter). Dissolved oxygen and pH were also measured, using a portable HACH (model DO175) dissolved oxygen meter and Orion field pH meter (model 210A, Orion Laboratories), respectively. A graduated rope with a heavy iron ring attached was used to measure water depth.

**Sampling for microbiological analysis.** Ten grams of each plant sample (roots of *Eichhorina crassipes* and *Pistia* spp. and the entire plant of *Lemna* spp.) was homogenized in 90 ml physiological saline (model 328179, Waring Product Division, Dynamics Corp, New Hartford, Connecticut). From 50 ml of each phytoplankton and zooplankton sample, 40 ml of unfixed sample was used for microbiological analysis after further concentration to 10 ml by filtering through a 20-μm mesh nylon filter and homogenizing in a Teflon-tipped tissue grinder (Wheaton Scientific, Millville, NJ) using a SteadFast stirrer (model 300, Fisher Scientific). Appropriate dilutions were used for plate counts. Ten grams of sediment were vortexed in 90 ml sterile physiological saline for 2 min and plated on mediums described below.

**Microbiological analysis. (i) *Vibrio cholerae*.** One ml of each zooplankton and phytoplankton homogenate was enriched in 10 ml (1X) alkaline peptone water. Ten ml of each plant homogenate and sediment sample was enriched in 5 ml triple strength alkaline peptone water (12). In addition, approximately 300 ml water, after filtration through a 20-μm plankton net, was sequentially filtered, using a 0.22-μm polycarbonate membrane (Millipore Corporation, Burlington, MA). The membrane was placed in 25 ml single-strength (1X) alkaline peptone water and incubated at 37°C for 6 h. After enrichment, appropriate dilutions were prepared and spread plated on thiosulfate citrate bile-salt-sucrose (TCBS) agar (Difco Laboratories) and tellurite taurocholate gelatin agar, prepared using ingredients from Difco Laboratories, and incubated at 37°C overnight. Colonies of presumptive vibrios were characterized using standard procedures (36, 39).

**(ii) Fecal coliforms.** Approximately 100 μl of appropriate dilutions of sediment was spread plated on fecal coliform (MFC) agar medium (Difco) (2). The plates were incubated at 44 ± 0.5°C for 18 to 24 h. Following incubation, characteristic blue colonies were counted as fecal coliforms. Plates with 30 to 300 colonies were selected for counting and counts were expressed as $\log_{10}$ CFU/g of sample. Tenfold dilutions of water samples were prepared in physiological saline and 0.1 ml was spread onto duplicate plates of MFC agar, following standard procedures (2).

**Preparation of colony blots.** Water samples were plated either directly or in 10-fold dilutions on nonselective LB agar plates with incubation overnight at 37°C. Culture plates containing 200 to 300 colonies were transferred to Whatman no. 541 filter paper and the filters were transferred colony side up onto no. 3 Whatman filters presoaked with lysis buffer (0.5 M NaOH, 1.5 M NaCl), followed by baking in a microwave oven for 2 min. The baked filters were placed on Whatman no. 3 filters soaked in 2 mol ammonium acetate buffer and maintained at room temperature for 5 min. Filters were rinsed twice in 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer, dried prior to storing at room temperature, and transported in batches to the University of Maryland for testing. The filters were treated with proteinase K solution (40 μg/ml in 100 ml 1× SSC) for 30 min at 42°C, with shaking, in a plastic container. Filters were rinsed three times in 1× SSC (100 ml) for 10 min at room temperature in a shaker water bath. Filters were dried and stored (27). As controls, the *V. cholerae* cholera toxin-positive (N16961) and -negative (NRT36S) strains were grown on

an L-agar plate and blotted on separate filters following the above method. During each hybridization batch, each of these blots was used as control blots.

**Cholera toxin oligonucleotide labeling.** For these studies we utilized a DNA oligonucleotide probe that has been shown to have essentially 100% sensitivity and specificity for the cholera toxin gene (*ctx*) in prior laboratory and field studies (13). Of particular note, the probe is drawn from a portion of *ctx* that is divergent from the *Escherichia coli* heat-labile toxin (LT) gene, permitting differentiation between strains carrying cholera toxin and heat-labile toxin genes (13).

The 5′-end labeling of the oligonucleotide (5′-CTC CGG AGC ATA GAG CTT GGA GG) was done using a 5′-end labeling kit (Boehringer Mannheim Corp) according to the manufacturer's protocol. Each 4 μl of oligonucleotide (80 pmol), 5 μl of 5× kinase buffer, 10 μl of [γ-$^{32}$P]ATP (Amersham Corp) (100 μCi), and 1 μl of T4 polynucleotide kinase enzyme (Gibco-BRL) were mixed in a reaction volume of 25 μl. The reaction was carried out at 37°C for 30 min and stopped by adding 2 μl of 0.5 M EDTA and 30 μl of Tris-EDTA and purified through a spin column (Probequant G-50, Phannacia Biotech) to remove unincorporated γ-$^{32}$P. The labeled oligonucleotide was used for hybridization (27, 40). Prehybridization was carried out in buffer containing 6× SSC, 1× Denhardt's solution, 1 mM EDTA containing 50 to 60 μl of heat-denatured salmon sperm DNA (10 mg/ml; Gibco BRL) in plastic bags, at 56°C, with moderate shaking (40 rpm) for 3 h. The buffer was removed and fresh buffer containing heat-denatured salmon sperm DNA (50 to 60 μl), and the probe ($10^7$ cpm) were added and incubation was continued at 56°C, with shaking at 40 rpm, overnight.

On the following day the filters were washed twice in 1X SSC/1% sodium dodecyl sulfate (SDS) buffer at 56°C for 10 min, with shaking at 60 rpm, followed by three washes in 1× SSC for 5 min each, with shaking, at room temperature. Filters were dried, exposed to X-ray films, and developed, and the total number of probe-positive colonies on the plate were counted. Filters were excluded from analysis if control filters (containing cholera toxin-positive and cholera toxin-negative *V. cholerae* strains) resulted in inappropriate results.

**Statistical analysis.** Clinical and environmental data collected through December 2000 were used to investigate the association between the number of cholera cases in each study area and environmental variables (8). A parsimonious statistical model using the smallest subset of the potential predictive environmental variables for cholera in humans was developed as follows. We examined lagged correlations between cholera cases and environmental variables and used stepwise regression to identify this subset. Variables that were significant in the stepwise regression and for which the direction of the estimated effect was consistent with expectation were included as covariates in a Poisson regression.

The outcome variable, log cholera incidence in humans, was regressed against the environmental predictors at various lags. The Poisson regression model for new cholera cases at time $t$, $\ln Y_t$, as a function of some lagged predictor at time $t - \tau$, $X_{t-\tau}$, is given by $\ln Y_t = \beta_0 + \beta_1 X_{t-\tau} + e+t$, where $\tau$ is the lag, the $\beta$'s are the regression coefficients, and $e_t$ is a random error.

The multiple regression for $k$ predictors is

$$\ln Y_t = \beta_0 + \beta_1 X_{1,t-\tau_1} + \beta_2 X_{2,t-\tau_2} + \ldots + \beta_k X_{k,1-\tau_k} + e_t,\ t \geq \max\{\tau_1, \tau_2, \ldots, \tau_k\}$$

where the lags for the $k$ predictors are $\tau_1, \tau_2, \ldots, \tau_k$. $Y_t$ was assumed to follow a Poisson distribution (23). A general correlation structure among the predictors was assumed for all the regressions; 95% confidence intervals for the risk ratio for each environmental variable were computed.

The fit of the Poisson regression models was examined by comparing observed and predicted numbers of cases in each area. For any fitted model, short-term predictions can be made up to the shortest lag $\tau^* = \min\{\tau_1, \tau_2, \ldots, \tau_k\}$. Thus, if we have data on the $k$ predictors up to time $t$, then we can predict cholera incidence up to time $t + \tau^*$. For example, the predicted number of cholera cases for time $t + \tau^*$ is

$$\ln \hat{Y}_{t+\tau^*} = \hat{\beta}_0 + \hat{\beta}_1 X_{1,t+\tau^*-\tau_1} + \ldots + \hat{\beta}_k X_{k,t+\tau^*-\tau_k}$$

To fit this predictive model for $Y_{t+\tau^*}$, a Bayesian framework and the Markov chain Monte Carlo approach were used (15). This approach also provides an estimate of the posterior predictive distribution, the upper 95th percentile of which provides an upper 95% prediction limit for the number of cholera cases. The interpretation is that, based on the observed data, the probability of the actual number of cholera cases $\tau^*$ months in the future falling below this limit is 0.95.

## RESULTS AND DISCUSSION

Approximately 10,800 water, plankton, sediment, and aquatic plant samples were collected during the course of this study. The percentages of samples positive for presence of copepods, cyanobacteria, fecal coliforms, genes coding for cholera toxin, and culturable *V. cholerae* are shown in Table 1. *Vibrio cholerae* O1 was cultured from 28 of 6,231 environmental samples. *V. cholerae* O139 was isolated from only one sample. The sites from which *V. cholerae* was isolated included 10 lakes, nine ponds, and 10 rivers, and the samples from these sites that yielded culturable *V. cholerae* were 13 water, seven phytoplankton, seven zooplankton, one water hyacinth, and one sediment sample.

TABLE 1. Surveillance of surface waters: percent of samples positive for copepods, cyanobacteria, fecal coliforms, gene for cholera toxin, and culturable *Vibrio cholerae* O1

| Variable | No. of samples | % positive |
|---|---|---|
| Copepods | 1,406 | 901 (64) |
| Cyanobacteria | 1,428 | 1,191 (83) |
| *ctx* probe | 1,346 | 245 (18) |
| Fecal coliforms | 1,384 | 1,311 (95) |
| *V. cholerae* O1 by culture | 6,231 | 28 (0.45) |

Descriptive statistics for the environmental variables used in the Poisson regressions are shown in Table 2. We found a very strong and consistent relationship between the ecological predictors and cholera incidence for Lake 2 in Bakerganj. Therefore, data from this water site were used in this report to examine the modeling approach. We did not find such consistency of prediction in the other sites; however, the general trends were relatively strong throughout all sites. The regression results indicated that several environmental variables were predictive of cholera cases in humans. In Bakerganj, water and air temperature, water depth, total rainfall, conductivity, dissolved oxygen, cholera toxin probe-positive count, and copepod counts in the surface water sites were significantly associated with the number of cholera cases. Table 3 shows the risk ratio (relative risk) estimates and 95% confidence intervals for significant environmental variables in the Bakerganj area.

**Influence of temperature.** At the Lake 2 site in Bakerganj, for a 5°C increase in the water temperature there was a 3.31-fold increase in the risk of cholera with a lag of 6 weeks. The 95% confidence interval for the relative risk is 2.38 to 4.59. That is, the risk of cholera is between 2.38 and 4.59 times

TABLE 2. Environmental variables monitored in this study from 1997 through 2000

| Variable | No. of samples | Median value | Minimum value | Maximum value |
|---|---|---|---|---|
| Conductivity (μS) | 1,205 | 149.00 | 22.00 | 400.00 |
| Copepods ($\log_{10}$ count/ml) | 1,406 | 1.80 | 0.00 | 4.40 |
| *ctx* probe-positive ($\log_{10}$ count/ml) | 1,346 | 0.70 | 0.00 | 4.00 |
| Water temp (°C) | 1,430 | 28.80 | 15.70 | 38.40 |
| Air temp (°C) | 1,432 | 28.60 | 10.50 | 38.50 |
| Water depth (ft)[a] | 1,423 | 6.50 | 1.00 | 50.00 |
| pH | 1,426 | 6.91 | 5.42 | 9.92 |
| Cyanobacteria ($\log_{10}$ count/ml) | 1,428 | 4.80 | 0.00 | 8.15 |
| Dissolved $O_2$ (mg/liter) | 1,116 | 3.45 | 0.00 | 12.00 |
| Fecal coliforms ($\log_{10}$ CFU/ml) | 1,384 | 1.30 | 0.00 | 4.48 |
| Salinity (ppt) | 1,400 | 0.10 | 0.00 | 0.80 |
| Total rainfall (mm/2 weeks) | 1,440 | 64.50 | 0.00 | 989.00 |

[a] One foot equals approx. 0.3 m.

TABLE 3. Risk ratio estimates and 95% confidence intervals for cholera incidence in Bakerganj

| Site | Variable (lag, in weeks) | Estimated risk ratio | 95% Confidence interval for risk ratio |
|---|---|---|---|
| River | Water temp (6) | 3.46 ($\Delta$ = +5°C) | 2.41, 4.97 |
| | Rainfall (8) | 1.73 ($\Delta$ = −50 mm) | 1.45, 2.07 |
| | Conductivity (2) | 1.16 ($\Delta$ = +150 μS) | 1.10, 1.23 |
| | Water depth (8) | 1.12 ($\Delta$ = −2 ft) | 1.06, 1.18 |
| Pond | Air temp (6) | 1.85 ($\Delta$ = +5°C) | 1.48, 2.30 |
| | Copepods (4) | 1.82 ($\Delta$ = +1 log) | 1.48, 2.24 |
| | Water depth (4) | 1.59 ($\Delta$ = −2 ft) | 1.24, 2.03 |
| | Dissolved $O_2$ (6) | 1.51 ($\Delta$ = +2 mg/dl) | 1.28, 1.77 |
| | Conductivity (2) | 1.48 ($\Delta$ = 150 μS) | 1.10, 1.98 |
| Lake 1 | Conductivity (2) | 1.85 ($\Delta$ = +150 μS) | 1.64, 2.08 |
| | Copepods (8) | 1.53 ($\Delta$ = +1 log) | 1.36, 1.73 |
| Lake 2 | Water temp (6) | 3.31 ($\Delta$ = +5°C) | 2.38, 4.59 |
| | Probe (0) | 3.09 ($\Delta$ = +1 log) | 2.24, 4.25 |
| | Conductivity (0) | 2.35 ($\Delta$ = +150 μS) | 1.60, 3.45 |
| | Rainfall (8) | 1.72 ($\Delta$ = 50 mm) | 1.45, 2.06 |

higher (with 95% confidence) 6 weeks after a 5°C increase in the water temperature. Figure 2 graphically illustrates this association. The plot shows a weak but statistically significant correlation of 0.22 between the number of cholera cases and water temperature with a lag of 6 weeks. At this same site, a 1 $\log_{10}$ increase in the number of bacteria that were *ctx* probe-positive was associated with a 3.09-fold increase in the risk of cholera, with a lag of 0 weeks (95% confidence interval for relative risk, 2.24 to 4.25) (Table 3, Fig. 3). Increases in water and air temperature were significantly associated with occurrence of cholera in all areas except Chaugachha. The three areas had significant associations for 11 out of 12 surface water samples. An increase of 5°C in temperature was associated with a risk ratio for cholera of 1.58 to 19.32, with the highest risk in Chhatak (≈19.3) and the lowest in Matlab (≈1.6). The lag periods ranged from 4 to 8 weeks.

Water temperature appears to have the most distinct relationship with cases of cholera in Matlab (Table 4). Similar observations were reported in earlier studies carried out in both the Bay of Bengal and the Chesapeake Bay (9, 24, 25). In Chhatak, increases in water temperature and air temperature and decreases in rainfall were followed by increases in cholera cases, with lags of up to 8 weeks (Table 5). In Chaugachha, increases in the number of bacteria that were *ctx* probe-positive were followed by increases in cholera cases, with a lag of approximately 4 to 8 weeks (Table 6).

**Rainfall and conductivity.** A close association was observed between rainfall and conductivity with cholera cases among villagers at the Lake 2 sampling site in Bakerganj (Fig. 4 and 5, respectively). It was interesting that, during the study, there was a negative association between the number of cholera cases and rainfall with a lag of 8 weeks (Fig. 4); with increased rain, the number of cholera cases declined. Decrease in salinity is most probably responsible, according to Louis et al. (25). Figure 5 illustrates a moderate, statistically significant correlation of 0.44 with no lag, indicating that increases in lake water conductivity are associated with simultaneous increases in cholera cases.

**Water depth.** The results for the other predictors and water sites were similarly informative. Decreases in pond water

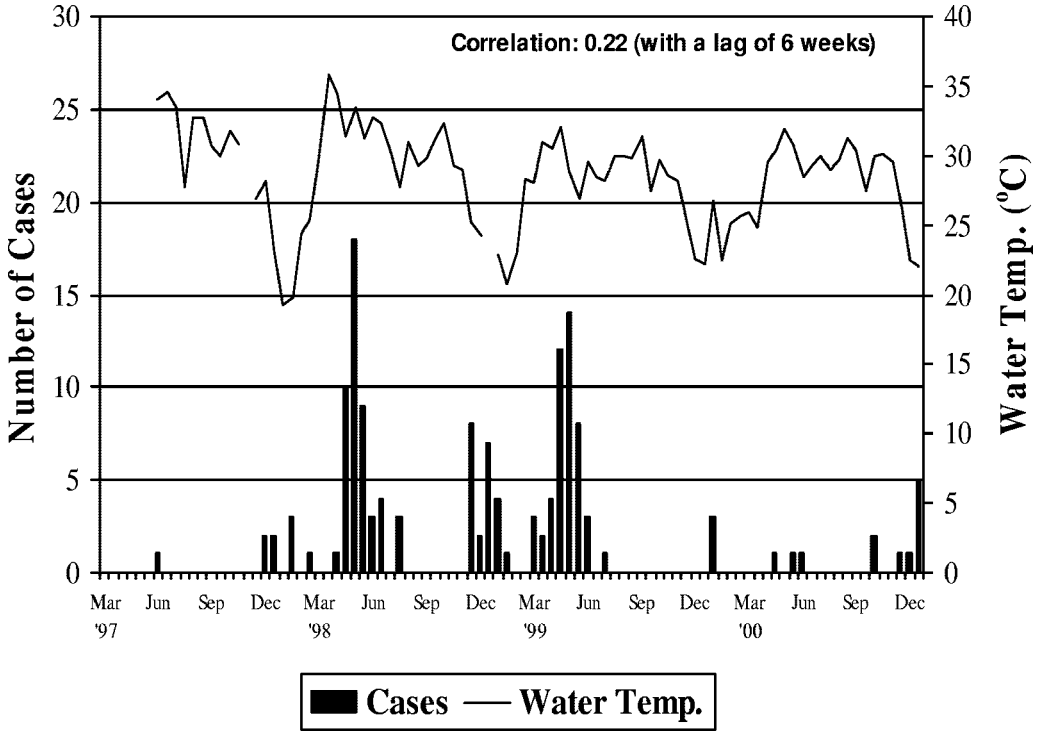


FIG. 2. Correlation between cases of cholera and water temperature in Bakerganj Lake 2.

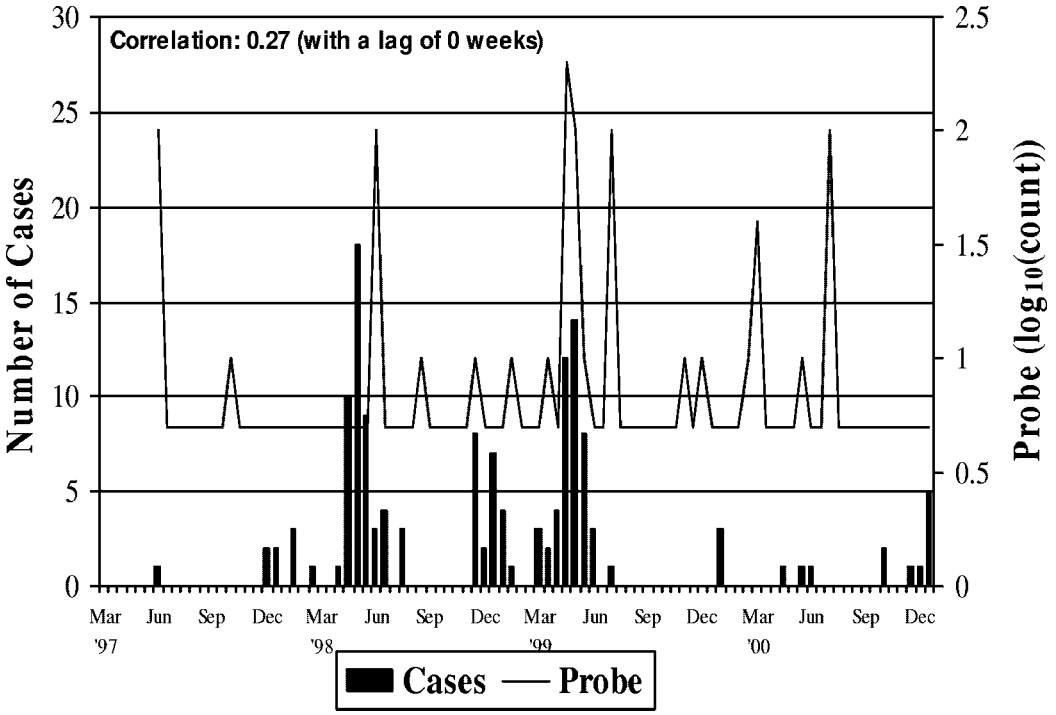


FIG. 3. Correlation between cases of cholera and detection of *ctx* gene in Bakerganj Lake 2.

depth in Bakerganj were followed by increases in cholera cases with a 4-week lag.

The effectiveness of the Poisson regressions used to obtain the Bakerganj Lake 2 risk ratios in Table 3 was evaluated by comparing the observed number of cholera cases at each time point with the predicted number of cases. Poisson regression models provide a reasonable preliminary approach for identifying environmental factors associated with cholera occurrence and for quantifying the risk of cholera for each factor, as shown in Fig. 6.

Based on epidemiological information, Matlab had the largest number of cholera cases, with an attack rate of 3.28 per thousand per year, while the designated control area (Chaugachha) had the fewest (29). In addition, all areas had *V. cholerae* O139 isolated from cholera cases arriving at the hospitals at some time during the surveillance, but with O1 vibrios predominant in all areas. During the period from November to December 1999, all four sites had simultaneous outbreaks of cholera. However, environmental conditions did not precisely correlate with each other in all of the geographic areas simultaneously.

**Cholera toxin probe result as a risk factor.** From each of the four surveillance sites and for the water samples, there was significant association between the physical and biological properties of the water samples and the occurrence of cholera. The risk factors varied, however, with water source and prop-

TABLE 4. Risk ratio estimates and 95% confidence intervals for cholera incidence in Matlab

| Site | Variable (lag in weeks) | Estimated risk ratio | 95% Confidence interval for risk ratio |
|---|---|---|---|
| River | Water temp (4) | 2.32 ($\Delta$ = +5°C) | 1.97, 2.73 |
| | Probe (0) | 1.42 ($\Delta$ = +1 log) | 1.20, 1.68 |
| | Copepods (0) | 1.19 ($\Delta$ = +1 log) | 1.08, 1.30 |
| Pond 1 | Water temp (4) | 1.58 ($\Delta$ = +5°C) | 1.38, 1.82 |
| | Copepods (4) | 1.27 ($\Delta$ = +1 log) | 1.14, 1.42 |
| Pond 2 | Water temp (4) | 1.82 ($\Delta$ = +5°C) | 1.60, 2.06 |
| | Probe (2) | 1.27 ($\Delta$ = +1 log) | 1.12, 1.44 |
| Lake | Water temp (4) | 1.59 ($\Delta$ = +5°C) | 1.34, 1.89 |
| | Probe (8) | 1.55 ($\Delta$ = +1 log) | 1.29, 1.85 |
| | Copepods (0) | 1.36 ($\Delta$ = +1 log) | 1.23, 1.50 |
| | Air temp (8) | 1.24 ($\Delta$ = +5°C) | 1.08, 1.42 |

TABLE 5. Risk ratio estimates and 95% confidence intervals for cholera incidence in Chhatak

| Site | Variable (lag in weeks) | Estimated risk ratio | 95% Confidence interval for risk ratio |
|---|---|---|---|
| River | Water temp (8) | 6.72 ($\Delta$ = +5°C) | 4.51, 10.02 |
| | Fecal coliforms (4) | 3.11 ($\Delta$ = +1 log) | 2.38, 4.06 |
| | Rainfall (2) | 2.08 ($\Delta$ = −50 mm) | 2.45, 1.76 |
| Pond 1 | Water temp (8) | 19.32 ($\Delta$ = +5°C) | 9.78, 38.18 |
| | Probe (0) | 1.70 ($\Delta$ = +1 log) | 1.32, 2.19 |
| | Rainfall (2) | 1.80 ($\Delta$ = −50 mm) | 2.13, 1.52 |
| Pond 2 | Air temp (8) | 3.89 ($\Delta$ = +5°C) | 2.91, 5.19 |
| | Fecal coliforms (4) | 2.52 ($\Delta$ = +1 log) | 1.70, 3.74 |
| | Rainfall (0) | 1.74 ($\Delta$ = −50 mm) | 2.00, 1.52 |
| Pond 3 | Air temp (8) | 3.91 ($\Delta$ = +5°C) | 3.04, 5.02 |
| | Rainfall (2) | 1.77 ($\Delta$ = −50 mm) | 2.04, 1.55 |

TABLE 6. Risk ratio estimates and 95% confidence intervals for cholera incidence in Chaugachha

| Site | Variable (lag in weeks) | Estimated risk ratio | 95% Confidence interval for risk ratio |
|---|---|---|---|
| River | Probe (2) | 2.77 ($\Delta$ = +1 log) | 1.88, 4.07 |
| Pond 1 | Probe (4) | 2.86 ($\Delta$ = +1 log) | 1.84, 4.44 |
| Pond 2 | Probe (4) | 2.67 ($\Delta$ = +1 log) | 1.89, 3.78 |
| Lake | Salinity (2) | 5.22 ($\Delta$ = +0.1 ppt) | 2.24, 12.17 |
| | Probe (8) | 2.73 ($\Delta$ = +1 log) | 1.83, 4.08 |
| | Copepods (4) | 1.75 ($\Delta$ = +1 log) | 1.20, 2.56 |

erties measured. Statistically significant risk ratios ranged from 1.12 to 19.32, with lags of 0 to 8 weeks. The *ctx* probe results, water and air temperature, water conductivity, copepod counts, and rainfall were the environmental factors that were most frequently associated with cholera cases. A one $\log_{10}$ increase in the number of bacteria that were *ctx* probe positive was found to be significantly associated with cholera in four surveillance areas, with the Bakerganj example shown in Fig. 3. The lake in Matlab revealed a one $\log_{10}$ increase in *ctx* probe results was associated with a 1.55-fold increase in the risk of cholera 8 weeks later. In Chaugachha, the probe was a predictor of cholera for all four water sampling sites, yielding risk ratios of 2.67 to 2.86, with a lag of 2 to 4 weeks.

It should be pointed out that the *ctx* probe was employed using colonies grown on nonselective medium and sent to the University of Maryland on filters for probing with $^{32}$P-labeled probes. Experiments involving radioactive chemicals could not be conducted in Dhaka and a nonradioactive probe was not available. Also, direct detection, without culture, had been planned but was unable to be done because of this limitation. Ongoing research is addressing this important factor and the results will be published elsewhere.

Because of the long lag period (several months between isolation and probe results), we were not able to pick probe-positive colonies from the original plates and, therefore, were not able to do further bacteriological studies of these organisms. The same probe but with a nonradioactive label was used in a study conducted in Lima, Peru, where all *ctx* probe-positive colonies were *V. cholerae*, confirmed by standard microbiological methods (13). Based on these results, and the exclusion from analysis of any filters lacking clear positive and negative control results, the probe data provide a reasonable estimate of total *ctx*-positive *V. cholerae* present in the samples examined. Obviously, direct detection without culture is both possible and preferable. These data are being gathered in the continuing research.

If a one-log increase can predict a cholera outbreak, it would confirm that the origin of the cholera-causing *V. cholerae* was ingestion via surface water used as drinking water. A positive *ctx* probe is important because it can provide direct association of those organisms carrying the gene as capable of causing an outbreak. Also, direct detection of *ctx*-carrying *V. cholerae* in the environment would provide an ever stronger correlation. In studies under way to follow up the observations and conclusions of this study are direct detection by fluorescent antibody and *V. cholerae*-specific probes to enumerate all *V. cholerae*, both culturable and nonculturable.

**Influence of plankton.** An increase of one $\log_{10}$ in copepod count was significantly associated with cholera cases for three lakes and two ponds in three of the four surveillance areas,

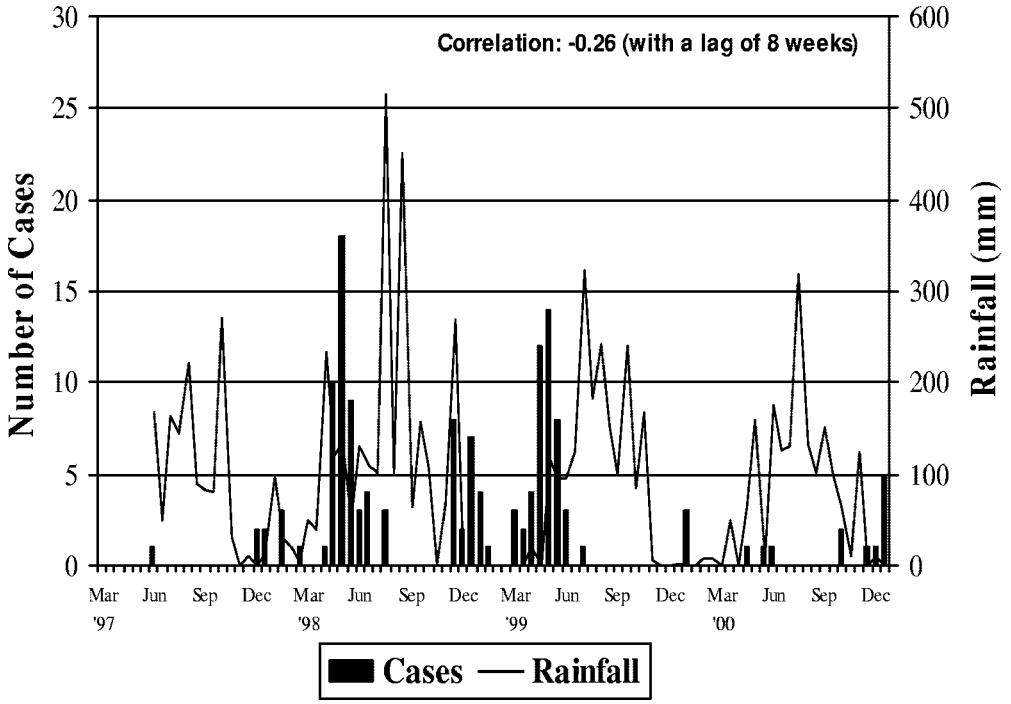


FIG. 4. Correlation between cases of cholera and rainfall in mm in Bakerganj area.

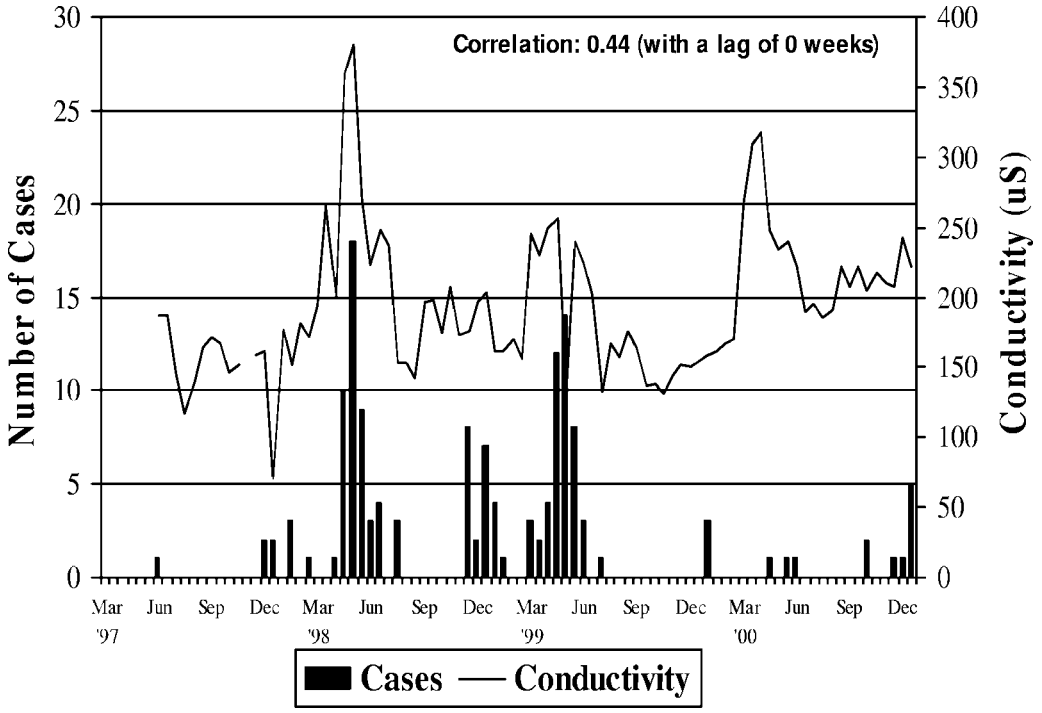


FIG. 5. Correlation between cases of cholera and conductivity measurements in Bakerganj Lake 2.

with a lag of 0 to 8 weeks. The total counts of phytoplankton were not found to be associated with the total number of cholera cases. In fact, the evidence points to the zooplankton as the significant factor. In Bakerganj, considering both lake and pond copepod data, the risk ratio was 1.36 to 1.73 and 1.48 to 2.30, respectively, and the lag time was 8 and 4 weeks, respectively.

**Other parameters or factors.** Another parameter that was found to be significantly associated with cholera cases was

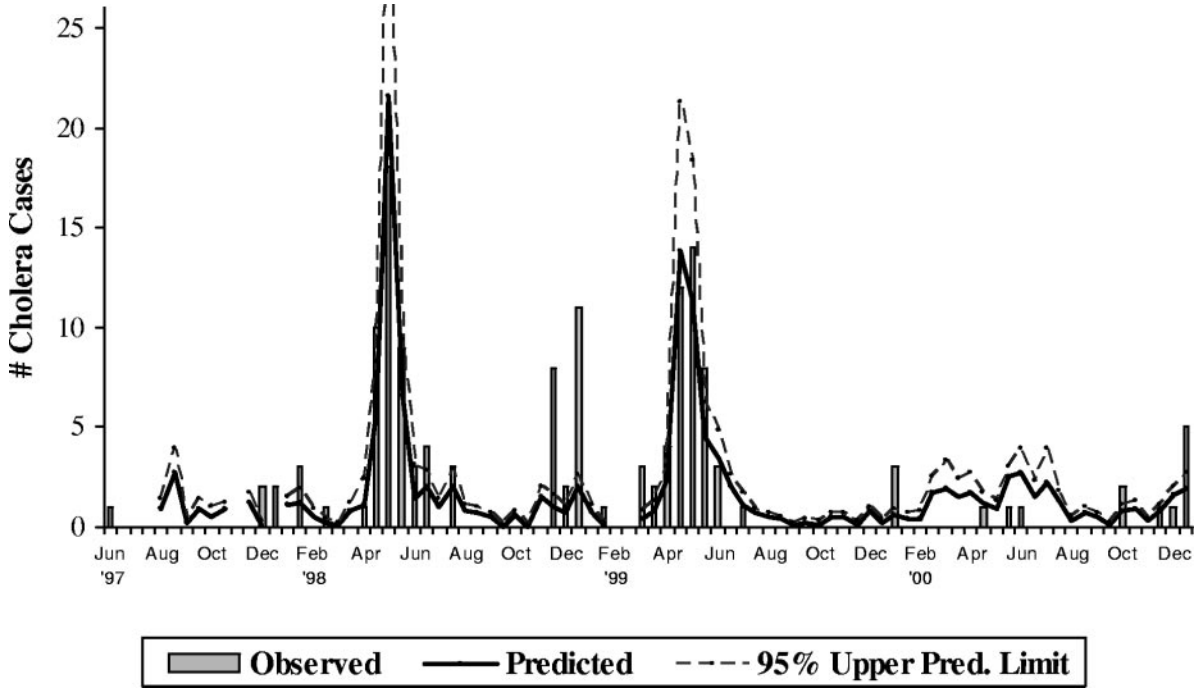


FIG. 6. Observed number of cholera cases versus number of cases predicted by the Poisson regression model and upper 95% prediction limit when using water temperature (lag, 6 weeks), *ctx* gene probe count (lag, 0 weeks), conductivity (lag, 0 weeks), and rainfall (lag, 8 weeks) for Lake 2 in Bakerganj.

conductivity. This association was observed for all four surface waters in Bakerganj, with a lag of 0 to 2 weeks, but not in the other sites in other areas. In this study, water depth, conductivity, rainfall, and copepod counts clearly had an impact on the occurrence of cholera in the cholera-endemic environment of Bangladesh.

From the results of this study, we were able to validate previous findings, notably the effect of water temperature on the occurrence of cholera. Previously a strong correlation was observed between sea surface temperature in the Bay of Bengal, measured by satellite remote sensing, and cases of cholera occurring in Dhaka, Bangladesh (9, 24). A remarkable finding from this study is that a nearly identical result was obtained for Bakerganj, with water temperature being directly correlated with cases of cholera (Fig. 2). Bakerganj is the southernmost site included in the study, having the most direct influence from the Bay of Bengal, where most of the initial cases of cholera occur, including the outbreak of newly recognized serotype O139 in 1992 (1).

Earlier, Colwell and Huq (11) and Louis et al. (25) proposed hypothetical models associating environmental factors with the occurrence of *V. cholerae* in the environment. Seasonal factors, such as rainfall and hours of sunlight, contribute directly to the physical and chemical characteristics of water that, in turn, affect plankton populations. Phytoplankton blooms are followed by zooplankton blooms, and copepods have been shown to have a *V. cholerae* flora (17, 20, 34). Thus, the time lag observed in this study for cholera cases, which was up to 8 weeks, is interpreted as the period of time during which the zooplankton bloom peaks. Further studies are under way to determine if there is a copepod species specificity with respect to *V. cholerae* occurrence in the environment.

The role of environmental factors in the occurrence and transmission of cholera is also being studied further to gain greater precision in the predictive model. Recently published analyses showed that temperatures of $\geq$25°C and pH of $\geq$7.0 enhance *Vibrio cholerae* counts in the Chesapeake Bay (21, 25). Earlier studies in Bangladesh and Peru identified temperature as a key factor associated with increased counts of *V. cholerae* and cases of cholera (9, 13, 24). The data presented here underscore the complexity of such models and highlight the importance of the multiple variables, temperature, salinity, and zooplankton, as key to the development of a predictive model for cholera.

In contrast, culturable *V. cholerae* (determined by conventional enrichment and selective media) do not provide useful information for prediction of cholera. Of the 10,798 environmental samples analyzed, *V. cholerae* O1 was cultured by conventional culture methods using TCBS plates only on 28 occasions (0.4%) and *V. cholerae* O139 on only one occasion, compared with 245 *ctx* probe-positive colonies on LB as required before transfer to the colony blot. In an earlier study, >63% of plankton samples were found positive for *V. cholerae* O1 by direct detection, using a fluorescent monoclonal antibody, while only 4% were culture positive (17). A recent report from Argentina showed none of 84 specimens yielded culturable *V. cholerae* O1 by conventional culturing methods, while 25% were positive by immunofluorescence direct microscopy and PCR (3). The failure of conventional microbiology with highly selective media to detect the organism clearly provides a warning with respect to public health.

In conclusion, the data gathered in this study further support the findings of numerous prior studies that environmental factors determine cholera epidemicity and that both quantitative and qualitative (genomic) data on *Vibrio* abundance in the environment are critical to estimate the risk of disease.

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