

Cholera in Lima, Peru, Correlates with Prior Isolation of *Vibrio cholerae* from the Environment

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The authors utilized a recently developed DNA probe technique to obtain quantitative data on occurrence of *Vibrio cholerae* in samples collected monthly from 12 environmental sites in Lima, Peru, from November 1993 through March 1995. Peak *V. cholerae* counts ranged from 10²/ml to 10⁵/ml, with the highest counts in sewage-contaminated areas and irrigation water. With our methodology, no *V. cholerae* cases were detected at any site during the winter months of July through October. Counts were detectable in the environment before onset of cholera in the community, with counts at "cleaner" sites upriver correlating significantly with occurrence of community disease 2 and 3 months later. In sites with heavy sewage contamination, *V. cholerae* could still be detected before the onset of cases in the community; however, in contrast to upriver sites, counts at these latter sites correlated most closely with the number of concurrently occurring cholera cases. These data support a model of cholera seasonality in which initial increases in number of *V. cholerae* in the environment (possibly triggered by temperature) are followed by onset of illness in the community, with these human cases further amplifying the organism as the epidemic cycle proceeds. *Am J Epidemiol* 1997;146:1067-75.

cholera; environment; *Vibrio cholerae*

Cholera is an ancient disease in the midst of a modern resurgence. In January 1991, for the first time in 100 years, Peru was struck by an explosive epidemic of cholera (1, 2); cases were first identified in several coastal cities, with rapid spread inland and across South and Central America. By the end of 1992, a total of 731,312 cases had been reported in 21 countries in the Western hemisphere (3). In subsequent years, cholera has settled into a typical seasonal pattern in the South American continent, with cases concentrated in the summer (January through March) in Lima and other areas of Peru.

An ability to anticipate the appearance of cholera in a population would have important public health im-

plications, allowing optimal allocation of resources for both prevention and treatment of disease. For this to happen, however, the ecology of the causative organism, *Vibrio cholerae*, must be understood. Within the last two decades, it has been recognized that the aquatic environment is a primary reservoir for *V. cholerae* (4). Studies in Bangladesh further suggest that when cholera season begins, cases occur almost simultaneously at multiple sites, with infecting strains having a multiplicity of epidemiologic markers (i.e., there is not a gradual, clonal spread of infection from one village to the next) (5). In keeping with these observations and in agreement with models suggested by other investigators (6, 7), we propose that an environmental trigger stimulates growth of *V. cholerae* in the environment at some time point before the beginning of "cholera season." The increasing numbers of organisms at multiple environmental sites then provide an increased opportunity for introduction of *V. cholerae* into human populations. Human cases, as they begin to occur, further amplify the organism through fecal contamination of food and water sources and recontamination of the environment, initiating the epidemic cycle.

Although the model described above is attractive (and has potential predictive utility), it remains largely untested. Due in part to technical difficulties in ob-

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Abbreviations: CFU, colony-forming units; CT, cholera toxin; CTAP, alkaline phosphatase-labeled oligonucleotide probe for cholera toxin gene; TCBS, thiosulfate citrate bile-salts sucrose.

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taining quantitative environmental cultures, no studies have systematically looked at numbers of environmental *V. cholerae* at multiple sites through an epidemic cycle and attempted to correlate these numbers with environmental parameters and the occurrence of human disease. Taking advantage of the recent availability of a sensitive and specific DNA probe for cholera toxin (CT)-producing strains of *V. cholerae* (8), we undertook such a study in the Lima metropolitan area in the 1993/1994 and 1994/1995 cholera seasons.

MATERIALS AND METHODS

Study design

The city of Lima has a population of approximately 6.5 million. Callao, to its west, is the main port of Peru, with a population of approximately 0.6 million. From November 1993 through March 1995, we collected environmental samples on a monthly basis from 12 sites in the Lima metropolitan area. Quantitative data from analysis of these samples were then compared with data on cholera cases obtained from the General Office of Epidemiology, Peruvian Ministry of Health.

As shown in figure 1, five of the 12 sites were along the Rímac River, which runs through the center of Lima. Site 1 was the most distant from the city (ap-

proximately 16 km east of downtown Lima), in a sparsely populated area; sites 2–5 were progressively downstream, with greater population densities. Sites 6 and 7 were from irrigation water in intensely cultivated vegetable farms approximately 12 km west of downtown Lima and 4 km north of Callao. Site 8 was along the Chillón River, which is approximately 20 km north of the city in a sparsely populated area. Sites 9–11 were sampled directly from sewage pipes that carry untreated sewage; site 12 was an untreated sewage lagoon in a sewage treatment plant approximately 18 km south of downtown Lima. Nonquantitative data on isolation of *V. cholerae* from samples collected at sites corresponding to our sites 9–12 have been reported by other investigators (9).

Environmental data were correlated with weekly data on numbers of cases of cholera in each of the five health subregions in metropolitan Lima (Lima-North, -East, -South, -City (central), and Callao). All hospitals and health centers in the province of Lima and Callao participated in reporting the number of suspect cholera cases to the General Office of Epidemiology, Ministry of Health, Lima. Suspect cholera was defined as acute diarrhea in a person presenting for treatment to a health facility. Central hospitals from each subregion cultured between 10 and 15 percent of the 18,728 suspect cases that occurred between November 1993 and March 1995, providing microbiologic confirmation of the diagnosis of cholera. The Peruvian National Institute of Health rechecked a 10 percent sample of strains initially screened by the subregions.

Environmental sampling

Surface water samples (approximately 200 ml) were collected at each site once a month. During the 17 months of the study, 200 samples were collected; four samples were missed (sites 1–4, January 1994). Water temperature and pH were determined on site with a digital thermometer and pH indicator strips (Color pHast indicator strips, pH 2–9, EM Reagents, Gills-town, New Jersey), respectively. Water salinity was determined in the laboratory by using a YSI model 33 S-C-T meter (Yellow Springs Instrument Co., Yellow Springs, Ohio), reading in parts per thousand.

Water samples were serially diluted in phosphate-buffered saline, and 100- or 200- μ l aliquots were spread on both selective (thiosulfate citrate bile-salts sucrose (TCBS)) and nonselective (Luria agar) media. Samples were processed within 30 minutes of collection, and plates were incubated at 37°C. Numbers of total culturable heterotrophic bacteria/ml were determined as the average of colony counts from four Luria agar plates, adjusted to take into account the sample dilution before plating.

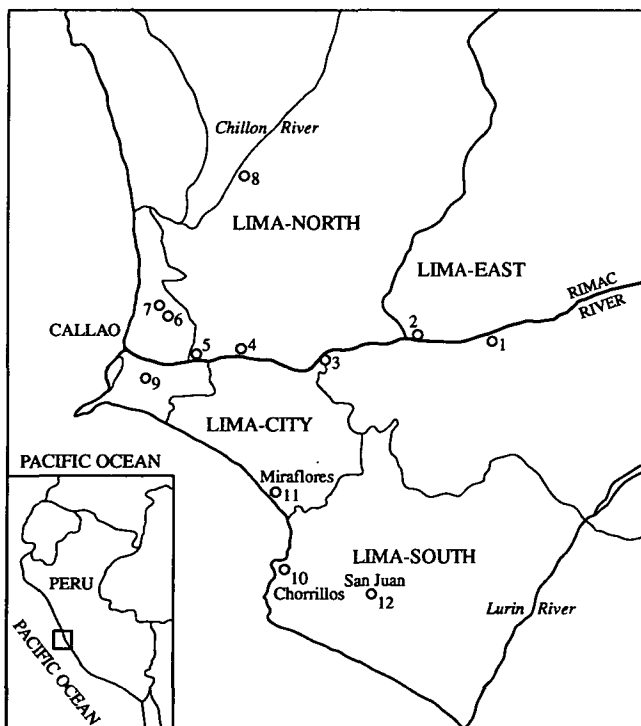


FIGURE 1. Metropolitan Lima. Sites indicating locations of sampling sites are: 1–5, Rímac River; 6 and 7, irrigation water in intensely cultivated vegetable farms; 8, Chillón River; 9–11, untreated sewage; 12, untreated sewage lagoon.

Detection of V. cholerae strains carrying the cholera toxin gene (DNA probe studies). Luria agar and TCBS plates with 20–500 colonies were overlaid with Whatman number 541 filters for colony blot hybridization with our nonradioactive, alkaline phosphatase-labeled oligonucleotide probe for the cholera toxin gene (CTAP) (8, 10). Colonies that hybridize with the gene probe appear as blue dots on the filter overlay. Filter preparation, hybridization, and probe development conditions have been reported previously (8). For this study, numbers of CT-positive *V. cholerae* in a sample were determined by averaging the number of probe-positive colonies from two to four filters and correcting for initial dilution of the sample. Depending on the number of bacterial colonies screened, this methodology will identify *V. cholerae* if it constitutes more than 0.05–1.0 percent of the total heterotrophic bacterial count at the site sampled.

To confirm probe specificity, efforts were made to recover probe-positive colonies from the original agar plates. Speciation of colonies picked in this way was confirmed by standard microbiologic techniques, as described below.

Detection of V. cholerae by standard microbiologic techniques. Twenty colonies were picked either from TCBS (based on a sucrose-positive phenotype) or Luria agar plates from the various sites. Each isolate was assayed for presumptive identification as *V. cholerae* based on reactions on triple sugar iron, lysine iron agar, citrate, urea, and sulfate indole motility slants. Oxidase-positive colonies were confirmed as *V. cholerae* O1 (the *V. cholerae* serotype responsible for the South American epidemic) by agglutination with Inaba-Ogawa polyvalent antiserum. Selected isolates identified by this procedure were also assayed by the CTAP probe.

Statistical methods

Because the distribution of cholera case counts, *V. cholerae* counts, and total bacterial counts are not normal, these variables were transformed for the purpose of analysis. The square root transformation of cholera case counts and the logarithmic transformation of *V. cholerae* and total bacterial counts were used in bivariate analysis, performed with Pearson correlation coefficients. The square root transformation of cholera case counts was also used for linear regression models, with this variable being the dependent variable. *V. cholerae* colony counts were transformed to dichotomous variables for the purpose of logistic regression analysis of the association between environmental factors and the appearance of *V. cholerae* in the water.

RESULTS

Comparison of probe and culture results

CT-positive (epidemic) *V. cholerae* cases were detected by the CTAP probe in 69 (34.5 percent) of the 200 samples collected. In contrast, nonquantitative conventional microbiologic techniques (i.e., selection of sucrose-positive colonies from TCBS media) yielded only 31 samples (15.5 percent of the total) that were positive for *V. cholerae* O1. With the CTAP probe, the number of CT-positive *V. cholerae* identified on nonselective media (Luria agar) ranged from 50 colony-forming units (CFU)/ml to 2×10^5 CFU/ml (geometric mean of approximately 3×10^4). In contrast, the number of *V. cholerae* O1 detected on selective media (TCBS), from the same samples, ranged from 0.5 CFU/ml to 250 CFU/ml (geometric mean of approximately 1.0×10^2).

Specificity of the CTAP probe was evaluated by isolation and microbiologic testing of probe-positive and probe-negative colonies from Luria agar and TCBS plates from probe-positive samples. In concurrent studies, *V. cholerae* colonies ($n = 87$) identified by standard microbiologic techniques from TCBS plates were screened with the probe. In all instances, there was 100 percent correlation between probe and microbiologic results. All CTAP-positive strains selected were identified biochemically as *V. cholerae* and serologically as serogroup O1.

Total culturable bacteria/CT-positive *V. cholerae*

When data from all sites were averaged for each month, there was minimal variation in the number of total culturable bacteria across time (figure 2). However, there was clear variation in number of total bacteria by site, with the lowest numbers seen in less contaminated areas upstream on the Rímac River (sites 1–3) and at the Chillón River site (site 8) (figure 3).

CT-positive *V. cholerae* were isolated at one or more sites each month between the months of November and June; cultures from July, August, September, and October were uniformly negative by both probe and standard microbiologic techniques. As shown in figure 4, peak monthly *V. cholerae* counts increased going downriver on the Rímac River (sites 1–5). The peak count on the Chillón River (site 8) was also low. Interestingly, some of the highest peak counts (exceeding those at three of the four sewage-associated sites) were in irrigation water at sites 6 and 7. Peak *V. cholerae* counts constituted, on average, 3.3 percent of total culturable heterotrophic bacteria, ranging from 0.4 percent in the sewage lagoon (site 12) to 7.5 percent at one of the irrigation sites (site 7).

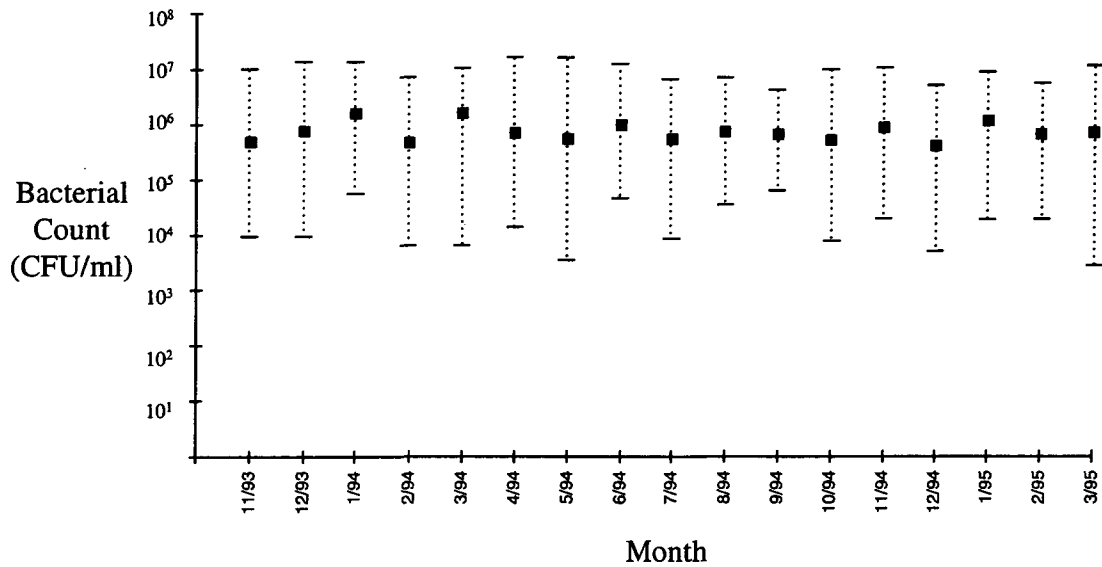


FIGURE 2. Geometric mean total bacterial counts in colony-forming units (CFU)/ml, for all sites, by month. Range shown by error bars.

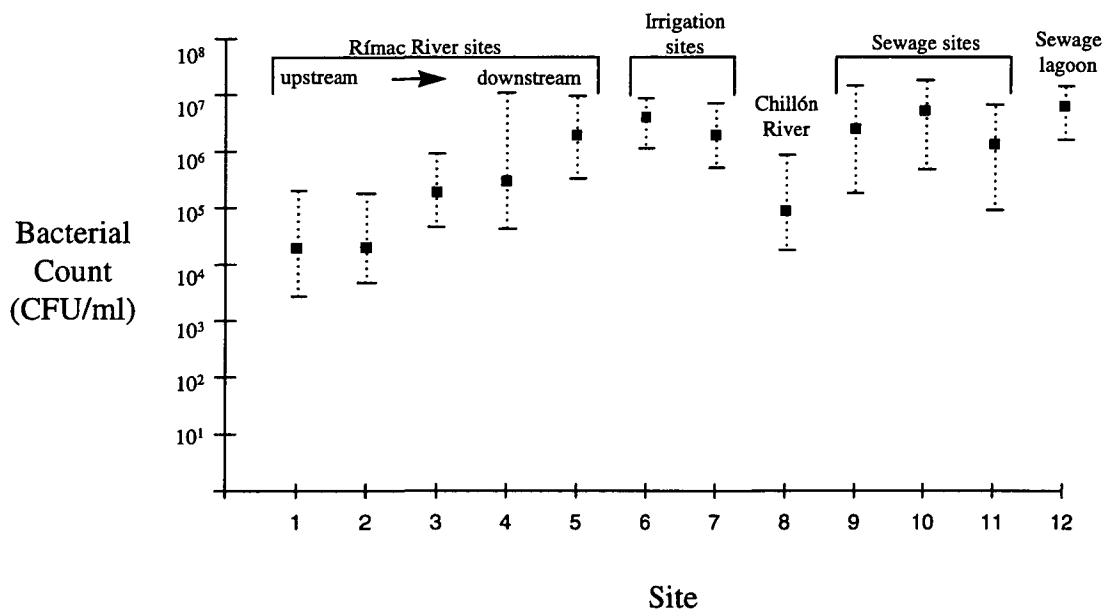


FIGURE 3. Geometric mean bacterial counts in colony-forming units (CFU)/ml for all months, by site. Range shown by error bars.

Environmental CT-positive *V. cholerae* and cholera in the community

In a simple bivariate analysis, there were significant correlations between the number of cholera cases in the community and counts of CT-positive *V. cholerae* in water; data for selected sites and time intervals are shown in table 1. For Rímac River sites (sites 1–5), counts of CT-positive *V. cholerae* tended to correlate most closely with the number of cholera cases in the community 2–3 months later. Data on isolation of CT-positive *V. cholerae* at site 1 (the farthest upriver

and presumably the “cleanest”) are included in figure 5, together with cholera case data for the Lima metropolitan area and east Lima, the subregion closest to the site. In the sewage-contaminated sites, CT-positive *V. cholerae* was again detectable in water before the major upslope of the cholera epidemic curve in the community; however, for these sites, the most significant correlation was between *V. cholerae* counts and concurrent cholera cases (figure 6: *V. cholerae* data from site 9, Callao sewage, overlaid on data on total cases in the Lima metropolitan area and in the subre-

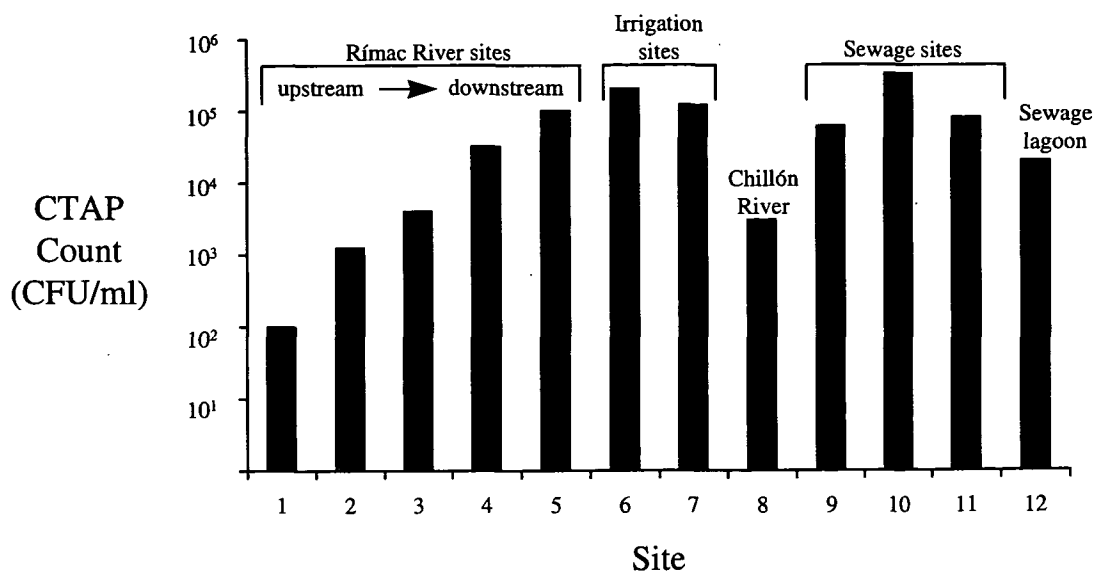


FIGURE 4. Peak *V. cholerae* counts (colony-forming units (CFU)/ml) as determined by oligonucleotide probe for cholera toxin gene (CTAP), by site.

TABLE 1. Correlation coefficients and *p* values for cholera case counts (as square root transformations) and environmental *Vibrio cholerae* counts (as logarithmic transformations) of samples collected in the metropolitan area of Lima, Peru, November 1993 through March 1995

Sites	Time of water collection relative to case counts									
	Concurrent	<i>p</i>	Months prior							
			1	<i>p</i>	2	<i>p</i>	3	<i>p</i>	4	<i>p</i>
1	-0.13	0.6	0.15	0.6	0.63	0.08	0.58	0.04	0.15	0.6
2	0.002	1.0	-0.01	1.0	0.33	0.3	0.44	0.1	0.09	0.8
3	-0.17	0.5	0.39	0.2	0.72	0.0004	0.47	0.1		
4	0.14	0.6	0.07	0.8	0.59	0.03	0.57	0.04	0.17	0.6
5	0.26	0.3	0.33	0.2	0.54	0.04	0.60	0.02	0.25	0.4
6	0.39	0.1	0.46	0.07	0.54	0.04	0.61	0.02	0.24	0.4
7	0.33	0.2	0.42	0.1	0.45	0.09	0.43	0.1	0.14	0.6
8	0.36	0.2	0.48	0.06	0.32	0.2	0.09	0.7	0.01	1.0
9	0.56	0.02	0.40	0.1	0.36	0.2	0.25	0.4	-0.20	0.5
10	0.46	0.07	0.35	0.2	0.50	0.06	0.47	0.09	0.09	0.8
11	0.50	0.04	0.43	0.1	0.44	0.1	0.40	0.2	-0.10	0.71
12	0.48	0.05	0.64	0.007	0.63	0.01	0.28	0.3	-0.25	0.4

gion of Callao). The results of analyses were similar when data on number of cholera cases in the subregion closest to each of the sites were substituted for data on total cholera cases in metropolitan Lima.

Regression analysis supports the results of the bivariate analysis. In table 2, various regression models for site 1 are included. Only when controlling for water temperature 4 months before occurrence of cholera cases (2 months before identification of *V. cholerae* in the environment) was there a reduction in the effect of CT-positive *V. cholerae* in the water on subsequent occurrence of cholera, with a corresponding loss of statistical significance. Total bacterial count was not significant as a variable in any of the models tested.

Environmental parameters and CT-positive *V. cholerae*

Seasonal changes in water temperature are shown in figure 7. Mean and range for pH and salinity determinations are presented in table 3.

Analysis of the relation between environmental parameters and CT-positive *V. cholerae* in the environment was restricted to sites that demonstrated an association between cholera cases and CT-positive *V. cholerae* counts in water in the preceding months (i.e., less contaminated sites). In bivariate analysis, the upper Rímac River sites (sites 1-3 combined) demonstrated a significant correlation between *V. cholerae* in the water and water temperature 2 months previously

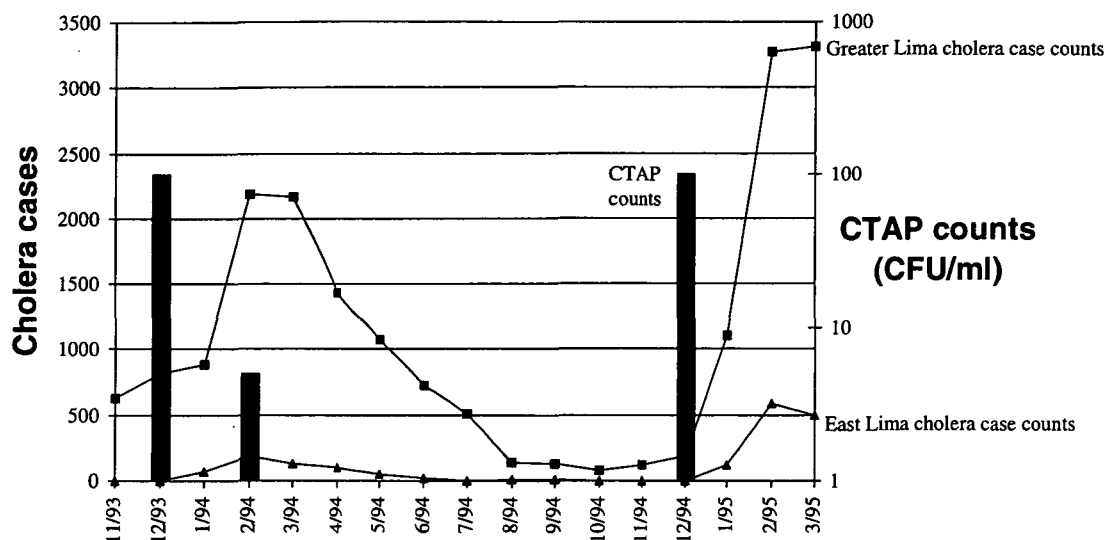


FIGURE 5. *V. cholerae* counts (colony-forming units (CFU)/ml) as determined by oligonucleotide probe for cholera toxin gene (CTAP) at site 1, by month. Data are overlaid on line graphs showing total number of cholera cases in metropolitan Lima (squares) and in east Lima (triangles). *, an environmental sample was not collected in January 1994.

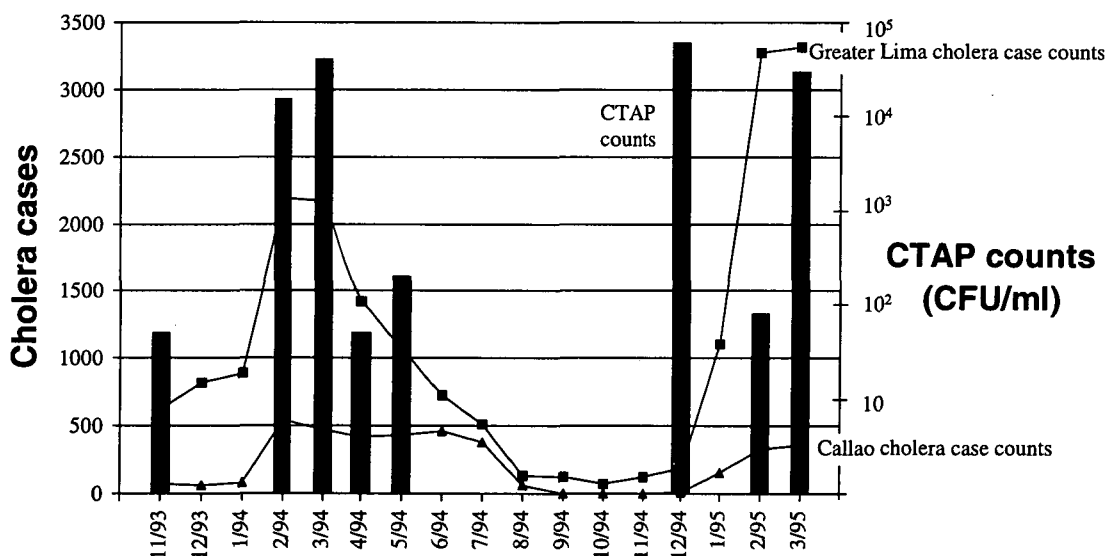


FIGURE 6. *V. cholerae* counts (colony-forming units (CFU)/ml) as determined by oligonucleotide probe for cholera toxin gene (CTAP) at site 9, by month. Data are overlaid on line graphs showing total number of cholera cases in metropolitan Lima (squares) and in Callao (triangles).

($r = 0.36$, $p = 0.02$). Site 8, on the Chillón River, demonstrated a similar correlation with temperature 2 months previously ($r = 0.52$, $p < 0.05$). Logistic regression analysis supported these associations. For sites 1–3, the odds ratio for the detection of *V. cholerae* in the water per degree increase in water temperature 2 months earlier was 1.4 (95 percent confidence interval 1.0–1.8). This association was unchanged when adjusting individually for concurrent total bacterial count, salinity 2 months previously, or pH 2 months previously (table 4). For site 8, the odds ratio for CT-positive *V. cholerae* in the water per degree of

increased water temperature 2 months earlier was 1.7 (95 percent confidence interval 0.9–3.1). As with sites 1–3, this association was unaltered when adjusted separately for concurrent water bacterial count, salinity 2 months previously, or pH 2 months previously (table 5).

DISCUSSION

This study was possible only because of the recent development of DNA probe techniques, which permit rapid, accurate quantitation of microorganisms (such as CT-positive *V. cholerae*) in the environment (10).

TABLE 2. Linear regression models for the association of total cholera cases in Lima (as square root transformations) with counts of *Vibrio cholerae* (cfu/ml) in the water at site 1 (as log transformations) from samples collected in the metropolitan area of Lima, Peru, November 1993 through March 1995

Model		Parameter estimate ± SE*	p value on parameter estimate	Adjusted R ² for model
1	Log CTAP* 2†	6.29 ± 2.25	0.02	0.34
2	Temp 4‡	4.78 ± 1.65	0.02	0.40
3	Log CTAP 2	2.83 ± 2.52	0.3	0.72
	Temp 4	5.23 ± 1.40	0.006	
4	Log CTAP 2	5.55 ± 2.43	0.04	0.33
	Log bacteria total§	-2.93 ± 3.43	0.4	
5	Log CTAP 2	3.25 ± 2.61	0.3	0.71
	Log bacteria total	2.55 ± 3.00	0.4	
	Temp 4	5.73 ± 1.55	0.008	

* SE, standard error; CTAP, alkaline phosphatase-labeled oligonucleotide probe for cholera toxin gene.

† Log transformation of *V. cholerae* counts (colony-forming units/ml) 2 months earlier.

‡ Water temperature (°C) 4 months earlier.

§ Log transformation of total bacterial count (colony-forming units/ml) 2 months earlier.

We utilized a nonradioactive oligonucleotide probe, developed in our laboratory, which is specific for the CT gene and which discriminates between CT and the heat labile toxin of enterotoxigenic *Escherichia coli* (8). In contrast to the many environmental *V. cholerae* strains that do not carry the gene for cholera toxin and consequently do not have the potential to cause epidemic disease (4, 11, 12), strains associated with epidemic cholera are uniformly CT positive. With our DNA probe, all colonies on a culture plate can be rapidly screened for the presence of CT-positive bac-

teria. Traditional microbiologic methods require the use of selective media, which, in our study, resulted in a decrease of two orders of magnitude in the number of *V. cholerae* colonies when compared with counts obtained with nonselective media. Furthermore, with traditional methods, it is necessary to pick "suspicious" colonies from the plate for further biochemical evaluation. Even with a skilled microbiologist picking 20 colonies from a plate for further evaluation, as was done in this study, less than half of the *V. cholerae*-positive samples were correctly identified with traditional methods.

Our data provide insights into the ecology of *V. cholerae* and its relation to occurrence of human disease. In upriver areas, we found that there was a significant correlation between counts of CT-positive *V. cholerae* cases in the environment and the number of cholera cases in the community 2–3 months later. In heavily populated areas/areas contaminated with sewage, *V. cholerae* counts correlated most closely with the number of concurrent cholera cases in the community; however, even in these areas, *V. cholerae* could be found in the environment before the onset of epidemic disease (13). These observations are in agreement with the recently published study by Madico et al. (9), who also found that *V. cholerae* O1 could be identified in sewage in Lima before occurrence of disease in the community. These observations do not support the hypothesis posed in the 1950s and 1960s that the presence of *V. cholerae* in the environment is solely the consequence of fecal contamination of the environment by ill individuals. They are compatible with the model presented earlier in this paper of *V.*

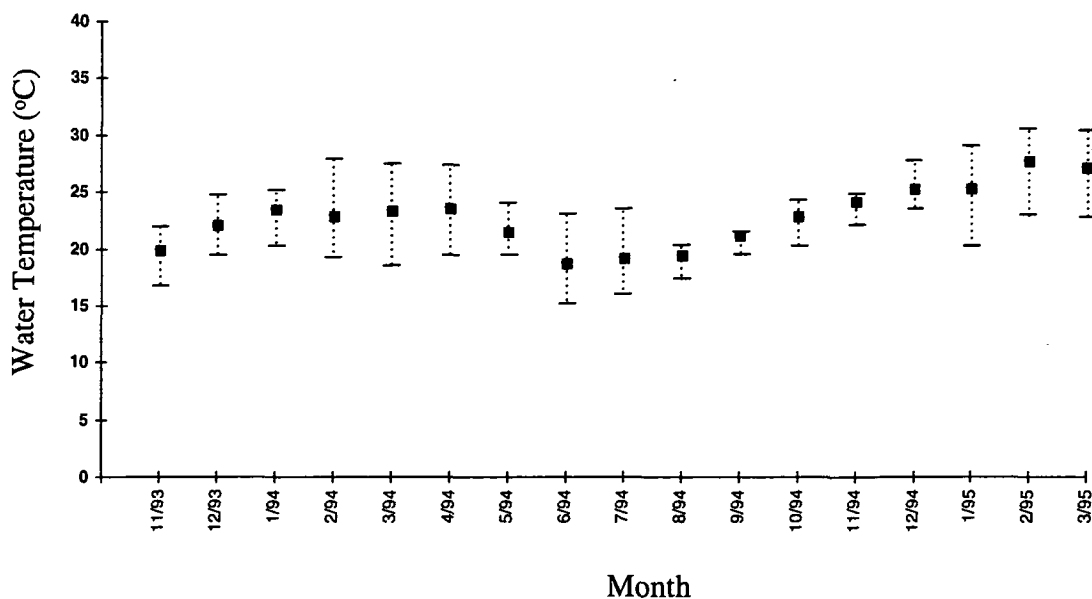


FIGURE 7. Mean water temperature for all sites, by month. Range shown by error bars.

TABLE 3. Mean and range for pH and salinity, by site, for *Vibrio cholerae* samples collected in the metropolitan area of Lima, Peru, November 1993 through March 1995

Site	pH		Salinity (ppt)	
	Mean	Range	Mean	Range
1	6.3	4.0–7.1	0.05	0.00–0.10
2	6.4	4.0–7.5	0.05	0.00–0.15
3	6.3	5.0–7.1	0.11	0.00–0.50
4	6.3	4.0–7.0	0.08	0.00–0.25
5	6.3	4.0–7.0	0.18	0.00–0.50
6	6.8	4.5–7.5	0.31	0.05–0.55
7	6.6	4.5–7.3	0.58	0.30–1.01
8	6.7	4.0–8.0	0.30	0.00–0.60
9	6.8	6.5–7.0	0.40	0.10–0.80
10	6.9	6.5–7.3	0.37	0.10–0.80
11	6.8	6.0–7.3	0.15	0.00–0.30
12	7.0	6.5–7.4	0.19	0.00–0.40

cholerae first increasing in number in its aquatic, environmental reservoir, and then beginning to spill over into human populations. At the same time, it is clear that fecal contamination of the environment serves to amplify the organism as the epidemic cycle proceeds. Persons with cholera may excrete 10^9 bacteria/ml of stool (4), providing ample opportunity for the increases in *V. cholerae* in sewage and sewage-contaminated environmental sites seen in conjunction with disease in the community.

We did not attempt to identify specific vehicles of transmission for cholera in the Lima metropolitan area. However, the counts that we observed within the city of Lima (approaching 10^5 CT-positive *V. cholerae*/ml of water at site 5 on the Rímac River) suggest that any use of untreated river water would carry a substantive risk of transmitting the organism. Even in “less contaminated” upriver areas, counts were in the range of 10–100 *V. cholerae*/ml, numbers that would be adequate to cause illness in at least a percentage of the population when administered in combination with food or in settings in which there was suppression of stomach acidity (14–16). Interestingly, we saw some of the greatest *V. cholerae* counts in water used to irrigate vegetables (which are often eaten without further cooking); it is likely that this was due, at least in part, to fecal contamination of the water, as reflected in the high total bacterial count in these samples. Studies in Chile have indicated that raw vegetables are an important source of enteric pathogens (17, 18). Our data suggest that similar risks exist in the Lima area (19) and underscore the ease and multiplicity of routes through which environmental organisms such as *V. cholerae* can move into human populations.

Work in Bangladesh has indicated that *V. cholerae* can assume a “viable but nonculturable” form, in which the organism remains alive (“viable”) but can-

TABLE 4. Logistic regression models for the association of *Vibrio cholerae* in the water (as dichotomous variables) with environmental factors for the upriver Rímac sites (sites 1, 2, and 3) from samples collected in the metropolitan area of Lima, Peru, November 1993 through March 1995

Model	Variable	Odds ratio	95% confidence interval
1	Temp 2*	1.4	1.0–1.8
2	Temp 2	1.5	1.0–2.1
	Log bacteria total	0.7	0.4–1.3
3	Temp 2	1.4	1.0–2.0
	Salt 2†	0.006	0–999
4	Temp 2	1.3	1.0–1.7
	pH 2‡	2.0	0.5–8.5

* Water temperature (°C) 2 months earlier.

† Water salinity 2 months earlier.

‡ Water pH 2 months earlier.

TABLE 5. Logistic regression models for the association of *Vibrio cholerae* in the water (as dichotomous variables) with environmental factors for the Chillón River site (site 8) from samples collected in the metropolitan area of Lima, Peru, November 1993 through March 1995

Model	Variable	Odds ratio	95% confidence interval
1	Temp 2*	1.7	0.9–3.1
2	Temp 2	1.9	0.9–3.9
	Log bacteria total	3.3	0.4–26.7
3	Temp 2	3.5	0.7–18.9
	Log bacteria total 3†	0.1	0.005–2.8
4	Temp 2	1.8	0.9–3.6
	Salt 2‡	14.2	0.03–999
5	Temp 2	1.9	0.9–4.1
	pH 2§	0.3	0.07–1.3

* Water temperature (°C) 2 months earlier.

† Log transformation of total bacterial count (colony-forming units/ml) 3 months earlier.

‡ Water salinity 2 months earlier.

§ Water pH 2 months earlier.

not be isolated on conventional media. These viable but nonculturable forms, as detected by direct immunofluorescence, may have counts that exceed viable counts by several orders of magnitude and may be present at times when no *V. cholerae* can be detected with standard culture techniques (4, 20, 21). In the laboratory, shifts between viable but nonculturable forms and culturable phenotypes may be triggered by changes in temperature and/or nutrients. Studies in Bangladesh have also identified possible symbiotic relations between *V. cholerae* and various species of plankton (20–22), with the suggestion that increases in numbers of *V. cholerae* in the environment are linked to seasonal plankton blooms.

Our probe (which identifies only those organisms that grow on culture media, i.e., culturable forms) was consistently unable to detect *V. cholerae* during colder, winter months (July through October). It is

possible that the appearance of *V. cholerae* at multiple sites in the early spring (November) was the result of human or animal contamination. It would appear more plausible, however, to hypothesize that the organism was present in the environment throughout the year, either in a culturable form at levels too low to be detected by the methodology used in this study or in a viable but nonculturable form. In this scenario, changing environmental conditions would trigger an increase in bacterial numbers and/or a shift from viable but nonculturable to culturable forms, permitting subsequent detection of the organism. Environmental parameters included in this study were limited to temperature, salinity, and pH; of these, the strongest correlation was with temperature. Additional studies are needed to confirm the presence of CT-positive *V. cholerae* in "culture-negative" months and to better delineate the environmental factors (including the presence or absence of plankton) that trigger the initial seasonal increase in counts.

In summary, our data suggest that there is a complex web of human and environmental interactions that lead to the seasonal epidemics of cholera seen in cholera-endemic regions. An understanding of these interactions may allow us to predict the onset (and potential intensity) of epidemics; it may also allow us to put in place interventions to limit or prevent occurrence of this potentially devastating illness.

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REFERENCES

- Centers for Disease Control. Cholera—Peru, 1991. *MMWR* 1991;40:108–10.
- Tauxe R, Seminario L, Tapia R, et al. The Latin American epidemic. In: Wachsmuth IK, Blake PA, Olsvik O, eds. *Vibrio cholerae* and cholera: molecular to global perspectives. Washington, DC: American Society for Microbiology, 1994.
- Centers for Disease Control. Update: cholera—Western hemisphere. *MMWR* 1993;42:89–91.
- Kaper JB, Morris JG Jr, Levine MM. Cholera. *Clin Microbiol Rev* 1995;8:48–86.
- Glass RI, Becker S, Huq MI, et al. Endemic cholera in rural Bangladesh, 1966–1980. *Am J Epidemiol* 1982;116:959–70.
- Miller CJ, Feachem RG, Drasar BS. Cholera epidemiology in developed and developing countries: new thoughts on transmission, seasonality, and control. *Lancet* 1985;1:261–3.
- Craig M. Time-space clustering of *Vibrio cholerae* O1 in Matlab, Bangladesh, 1970–1982. *Soc Sci Med* 1988;26:5–13.
- Wright AC, Guo Y, Johnson JA, et al. Development and testing of a non-radioactive DNA oligonucleotide probe that is specific for *Vibrio cholerae* cholera toxin. *J Clin Microbiol* 1992;30:2302–6.
- Madico G, Checkley W, Gilman RH, et al. Active surveillance for *Vibrio cholerae* O1 and vibriophages in sewage water as a potential tool to predict cholera outbreaks. *J Clin Microbiol* 1996;34:2968–72.
- Wright AC, Hill RT, Johnson JA, et al. Distribution of *Vibrio vulnificus* in the Chesapeake Bay. *Appl Environ Microbiol* 1996;62:717–24.
- Minami A, Hashimoto S, Abe H, et al. Cholera enterotoxin production in *Vibrio cholerae* O1 strains isolated from the environment and from humans in Japan. *Appl Environ Microbiol* 1991;57:2152–7.
- Morris JG Jr. Non-O group 1 *Vibrio cholerae*: a look at the epidemiology of an occasional pathogen. *Epidemiol Rev* 1990;12:179–91.
- Ventura G, Roberts L, Gilman R. *Vibrio cholerae* non-O1 in sewage lagoons and seasonality in Peru cholera epidemic. *Lancet* 1992;339:937–8.
- Cash RA, Music SI, Libonati JP, et al. Response of man to infection with *Vibrio cholerae*. I. Clinical, serologic, and bacteriologic responses to a known inoculum. *J Infect Dis* 1974;129:45–52.
- Levine MM, Black RE, Clements M-L, et al. Volunteer studies in development of vaccines against cholera and enterotoxigenic *Escherichia coli*: a review. In: Holme T, Holmgren J, Merson MM, et al, eds. *Acute enteric infections in children: new prospects for treatment and prevention*. New York, NY: Elsevier/North Holland Biomedical Press, 1981.
- Sack GH, Pierce NF, Hennessey KN, et al. Gastric acidity in cholera and noncholera diarrhoea. *Bull World Health Org* 1972;47:31–6.
- Levine MM. South America: the return of cholera. *Lancet* 1991;338:45–6.
- Hopkins RJ, Vial PA, Ferreccio C, et al. Seroprevalence of *Helicobacter pylori* in Chile: Vegetables may serve as one route of transmission. *J Infect Dis* 1993;168: 222–6.
- Anonymous. Of cabbages and chlorine: cholera in Peru. *Lancet* 1992;340:20–1.
- Huq A, Colwell RR, Rahman R, et al. Detection of *Vibrio cholerae* O1 in the aquatic environment by fluorescent-monoclonal antibody and culture methods. *Appl Environ Microbiol* 1990;56:2370–3.
- Xu HS, Roberts NC, Adams LB, et al. An indirect fluorescent antibody staining procedure for detection of *Vibrio cholerae* serovar O1 cells in aquatic environmental samples. *J Microbiol Methods* 1984;2:221–31.
- Huq A, Small EB, West PA, et al. The role of planktonic copepods in the survival and multiplication of *Vibrio cholerae* in the aquatic environment. In: Colwell RR, ed. *Vibrios in the environment*. New York, NY: John Wiley and Sons, 1984.