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Active Surveillance for *Vibrio cholerae* O1 and Vibriophages in Sewage Water as a Potential Tool To Predict Cholera Outbreaks

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The 1991 Peruvian cholera epidemic has thus far been responsible for 600,000 cholera cases in Peru. In an attempt to design a cholera surveillance program in the capital city of Lima, weekly sewage samples were collected between August 1993 and May 1996 and examined for the presence of *Vibrio cholerae* O1 bacteria and *V. cholerae* O1 bacteriophages (i.e., vibriophages). During the 144 weeks of surveillance, 6,323 cases of clinically defined cholera were recorded in Lima. We arbitrarily defined an outbreak as five or more reported cases of cholera in a week. The odds of having an outbreak were 7.6 times greater when *V. cholerae* O1 was present in sewage water during the four previous weeks compared with when it was not ($P < 0.001$). Furthermore, the odds of having an outbreak increased as the number of *V. cholerae* O1 isolations during the previous 4 weeks increased ($P < 0.001$). The odds of having an outbreak were 2.4 times greater when vibriophages were present in sewage water during the four previous weeks compared with when they were not, but this increase was not statistically significant ($P = 0.15$). The odds of having an outbreak increased as the number of vibriophage isolations during the previous 4 weeks increased ($P < 0.05$). The signaling of a potential cholera outbreak 1 month in advance may be a valuable tool for the implementation of preventive measures. In Peru, active surveillance for *V. cholerae* O1 and possibly vibriophages in sewage water appears to be a feasible and effective means of predicting an outbreak of cholera.

Vibrio cholerae O1 has been responsible for 600,000 cases in Peru since the beginning of the 1991 cholera epidemic (14). During this epidemic, the Peruvian Ministry of Health (PMOH) focused on prevention and treatment of cholera by active case finding, health education, chlorination, distribution of bottled water, emergency cholera treatment wards, and rehydration and antimicrobial therapy. In spite of these aggressive measures, routes of transmission remained poorly identified, and *V. cholerae* O1 disseminated rapidly throughout the country (9).

The role of various water sources in the spread of cholera has been well documented since John Snow epidemiologically linked Thames River water from the Broad Street pump and cholera cases in London in 1855 (16). In a South African mine, *V. cholerae* O1 was isolated from sewage water prior to and during an outbreak of cholera (10). In Peru, *V. cholerae* O1, not detected in sewage surveillance prior to 1991, has since been isolated from water sources, including sewage runoff, rivers, and seawater (18).

In countries where cholera is endemic, *V. cholerae* O1 bacteriophages (i.e., vibriophages) have been detected in sewage water (2, 13) and have served as strain markers (1) and for typing of *V. cholerae* O1 strains (13). The presence of vibriophages in water contaminated with *V. cholerae* O1 depends on the ability of the vibriophage to infect and lyse these bacteria (4).

In countries where cholera exhibits a seasonal behavior characterized by fluctuations in incidence (17), environmental surveillance can play an important role in cholera control. Asymptomatic infection with *V. cholerae* O1 occurs much more frequently than do active cases (19), and surveillance by detecting *V. cholerae* O1 bacteria and vibriophages in sewage water may be a feasible means of predicting outbreaks of cholera before a significant number of cases occur. In this study, we explore the relationship between the presence of *V. cholerae* O1 and vibriophages in sewage water, weekly ambient temperature, and the weekly number of cholera cases in the city of Lima.

MATERIALS AND METHODS

Study setting. The study was conducted in the capital city of Lima (population, 7 million), located in a desert region of the Peruvian coastline. Precipitation averages 20 mm/year, and the local temperature varies between 15 and 25°C (Peruvian Weather Service).

Sampling procedure. With the help of the Peruvian Water Company, the sea outlets of three of the city's major sewer lines were chosen as sewage collection sites. A raw sewage oxidation-purification pond in the district of Pampas de San Juan in the southern section of the city was selected as a fourth sample site (12). The four sample sites defined a surveillance area which monitored the northern (Callao), central (Miraflores), and southern sections (Chorillos) of Lima. This area was extended in November 1993 to include three additional sewer lines located in Pampas de San Juan de Miraflores.

Between August 1993 and May 1996, one field worker collected sewage samples from each site every Monday. The sewage samples were collected between 8 a.m. and 12 p.m. following a strict time schedule to prevent processing delays. Each sample was collected into a sterile bottle. The field worker began the sample enrichment at the sewage site. A 1:1 volume of 250 ml of sewage and alkaline peptone broth (APB) (Difco Laboratories, Detroit, Mich.) was mixed and incubated for 20 h at room temperature. All samples were processed at Cayetano Heredia, except for the Miraflores samples, which were processed at the Peruvian National Institute of Health.

***V. cholerae* isolation.** Enriched specimens were cultured on selective agar (thiosulfate-citrate-bile salt-sucrose agar) for 24 h at 37°C. Isolated colonies that agglutinated with antisera to *V. cholerae* O1 were then confirmed as *V. cholerae* O1 either by a commercial coagglutination test (New Horizons Diagnostic Corp., Columbia, Md.) (3) or by biochemical testing (8). *V. cholerae* O1 isolates were identified as the Inaba or Ogawa serotype by a latex serological test (Denka Seiken, Tokyo, Japan) (3).

Vibriophage isolation. Vibriophages were detected by a modification of the technique described by Chattopadhyay et al. (4). Water samples were mixed with APB in a 2-ml:5-ml (vol/vol) ratio and inoculated with a 1-ml cell suspension of

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an overnight culture in APB of an Ogawa serotype *V. cholerae* O1 El Tor biotype strain (LPS2) and the last *V. cholerae* O1 isolated as the amplifying strain (20). This strain was added to the procedure after May 1995 because of a change in the phage receptor. The LPS2 strain was isolated in our laboratory in July 1993 from sewage water obtained from an oxidation-purification pond in the district of Pampas de San Juan. This mixture was incubated at 37°C for 24 h to amplify the number of phages. The APB-water sample mixture was then centrifuged at 2,000 rpm (1,700 × *g*) for 15 min, and the supernatant was retained. The APB-water supernatant was then filtered with a sterile 0.22-μm-pore-size filter to obtain a bacterium-free solution for phage testing (4). Immediately after *V. cholerae* O1 LPS2 and the most recent strain were streaked on a Trypticase soy agar (Difco Laboratories) culture plate, 20 μl of the filtrate was inoculated on the plate. Six samples of filtrates were inoculated per Trypticase soy agar plate, and each plate was incubated for 24 h at 37°C. A plate was positive for vibriophages when either a lytic or lysogenic plaque type was observed. A positive sample of sewage water was tested concurrently with the new sample as a control.

Specificity testing of phages. If more than one site was positive for vibriophages in the same week, only one of the positive samples was randomly selected for specificity testing. Bacterium-free filtrates of vibriophages were purified by picking single-plaque isolations until homogeneous plaques of lysis against the *V. cholerae* O1 El Tor biotype LPS2 propagating strain were obtained. Phage lysates were prepared in APB with LPS2. Vibriophage preparations were tested for lysis against (i) 13 strains of *V. cholerae* O1 El Tor biotype and 1 strain of the classic biotype, (ii) 9 strains of *V. cholerae* non-O1, (iii) three other *Vibrio* species (i.e., *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, and *Vibrio campbellii*), and (iv) 1 strain each of 13 different bacteria (i.e., *Pseudomonas aeruginosa*, an *Enterobacter* sp., *Escherichia coli*, *Proteus vulgaris*, *Salmonella bareilly*, *Salmonella typhi*, a *Yersinia* sp., *Bacillus subtilis*, *Bacillus anthracis*, *Staphylococcus epidermidis*, *Aeromonas hydrophila*, a *Klebsiella* sp., and *Citrobacter freundii*). All *Vibrio* strains were kindly provided by D. N. Taylor (Naval Medical Research Institute Detachment, Lima, Peru), and the non-*Vibrio* species were kindly provided by the late A. Yi (Microbiology Department, Cayetano Heredia University, Lima, Peru).

Data collection. Weekly reports of new cholera cases in the city of Lima were obtained from the PMOH. Case reporting to the PMOH comprised new cases from all of the city's health-care facilities (including hospitalized patients). The PMOH's report was derived from either clinical diagnosis or laboratory confirmation. The weekly air temperature was obtained from the Peruvian Weather Service's atmospheric station.

Definitions. A clinical case was defined by the PMOH as the presence of watery diarrhea with moderate to severe dehydration in individuals older than 5 years of age. A confirmed case was defined as occurring in a patient with a positive culture for *V. cholerae* O1. A hospitalized patient was defined as one with a clinical or confirmed case who stayed in a health-care facility for longer than 12 h (5, 20). A week was considered positive for *V. cholerae* O1 or vibriophages if at least one of the sites tested positive for either organism. We arbitrarily defined an outbreak of cholera as five or more new cases in a week.

Statistical analysis. We used a chi-square test to measure the association between the presence of *V. cholerae* O1 and vibriophages in sewage water during the same week and a correlation coefficient to measure the linear association between weekly ambient temperatures and the number of weekly cholera cases.

Each study week was classified by ambient temperature (>20.0 and ≤20.0°C) and by the presence or absence of *V. cholerae* O1 or vibriophages in sewage water. An odds ratio was used to measure the relationship between *V. cholerae* O1 or vibriophages in sewage water and weekly ambient temperatures >20.0 or ≤20.0°C in the same week. We fitted this odds ratio with a logistic regression model, using the presence or absence of *V. cholerae* O1 or vibriophages in sewage water as the dependent variable and an indicator variable for weekly ambient temperatures >20.0°C as the independent variable.

To measure the relationship between an outbreak of cholera in the city and *V. cholerae* O1 or vibriophages in sewage water, we used the odds ratio. This ratio measured the odds of having an outbreak of cholera when *V. cholerae* O1 or vibriophages were present in sewage water in the previous 4 weeks compared with when they were not. Based on our arbitrary definition of an outbreak, we classified the study weeks into one of the following: weeks with five or more cases and weeks with fewer than five cases. For each week, we examined both the presence and the number of positive *V. cholerae* O1 or vibriophage isolations in sewage water during the previous 4 weeks. To estimate the odds ratio of having an outbreak when *V. cholerae* O1 was present in sewage water during the previous 4 weeks, we fitted three separate logistic regression models using the occurrence of an outbreak as the dependent variable, the occurrence of an outbreak in the previous week as an independent variable, and one of the following as the second independent variable; (i) the presence of *V. cholerae* O1 or vibriophages in sewage water during the previous 4 weeks or (ii) the number of positive *V. cholerae* O1 or vibriophage isolations in sewage water during the previous 4 weeks or (iii) an indicator variable for ambient temperatures >20.0°C. We included the presence of an outbreak in the previous week as an independent variable in the logistic regression model to control for the serial dependence of the observations (i.e., the presence of an outbreak may depend on the presence of an outbreak in the previous week). A limiting factor in our analysis is that the serial dependence of the observations may be different from that from a first-order autoregressive structure.

RESULTS

In 144 weeks of surveillance, 780 sewage samples were collected. *V. cholerae* O1 was isolated in 28% (41 of 144) of the surveillance weeks and in 9% (69 of 780) of the sewage samples; vibriophages were isolated in 16% (23 of 144) of the surveillance weeks and in 5% (35 of 780) of the sewage samples (Fig. 1). There was a strong association between the isolation of *V. cholerae* O1 and vibriophages in sewage water in the same week ($P < 0.01$ by the chi-square test). The vibriophages isolated from this study appeared to be specific for *V. cholerae* O1 El Tor biotype only; each of 19 vibriophage isolates selected for specificity testing lysed the 13 strains of *V. cholerae* O1 El Tor. No lysis was evident, however, when these vibriophages were tested against a classic *V. cholerae* strain, nine non-O1 *V. cholerae* strains, 3 other *Vibrio* species, or the 13 non-*Vibrio* species tested.

Between August 1993 and May 1996, the PMOH recorded 6,323 clinical cases and seven deaths by cholera in the city of Lima. In 34% (2,154 of 6,323) of these cases the patients were hospitalized. Fifty-six percent (1,052 of 1,891) of a subsample of patients (1,891 of 6,323) with clinically defined cholera were culture positive for *V. cholerae* O1.

V. cholerae O1 and vibriophages appeared to be isolated more frequently when the weekly ambient temperature was >20.0°C (Fig. 2). The odds of isolating *V. cholerae* O1 from sewage water were 7.6 times greater when the ambient temperature was >20.0°C compared with when the ambient temperature was ≤20.0°C ($P < 0.001$ by logistic regression). The odds of isolating vibriophages in sewage water were 1.5 times greater when the ambient temperature was >20.0°C compared with when the ambient temperature was ≤20.0°C, but this increase was not statistically significant ($P = 0.38$ by logistic regression).

The odds of having an outbreak were 7.6 times greater when *V. cholerae* O1 was isolated from sewage water at least once during the previous 4 weeks compared with when it was not ($P < 0.001$ by logistic regression). As shown in Table 1, the chance of having an outbreak increased as the number of positive *V. cholerae* O1 isolations in sewage water during the previous 4 weeks increased ($P < 0.001$ by logistic regression).

The odds of having an outbreak were 2.4 times greater when vibriophages were isolated from sewage water at least once during the previous 4 weeks compared with when they were not, but this increase was not statistically significant ($P = 0.15$ by logistic regression). As shown in Table 2, the chance of having an outbreak increased as the number of positive vibriophage isolations in sewage water during the previous 4 weeks increased ($P < 0.05$ by logistic regression).

There was a significant positive correlation between the number of weekly cholera cases and the weekly ambient temperature ($r = 0.52$; $P < 0.001$). The odds of having an outbreak were 3.5 times greater when the ambient temperature was >20.0°C compared with when it was ≤20.0°C ($P < 0.05$ by logistic regression).

DISCUSSION

Previous studies have shown that *V. cholerae* O1 survives poorly in water at temperatures below 10°C (15) and actively proliferates in nutrient-rich waters at temperatures above 20°C (5, 11). In our study, we found that ambient temperatures greater than 20°C were associated with increased numbers of *V. cholerae* O1 bacteria in sewage samples and documented cholera cases. Water temperature is preferred over ambient temperature to study *V. cholerae* in the environment. In our

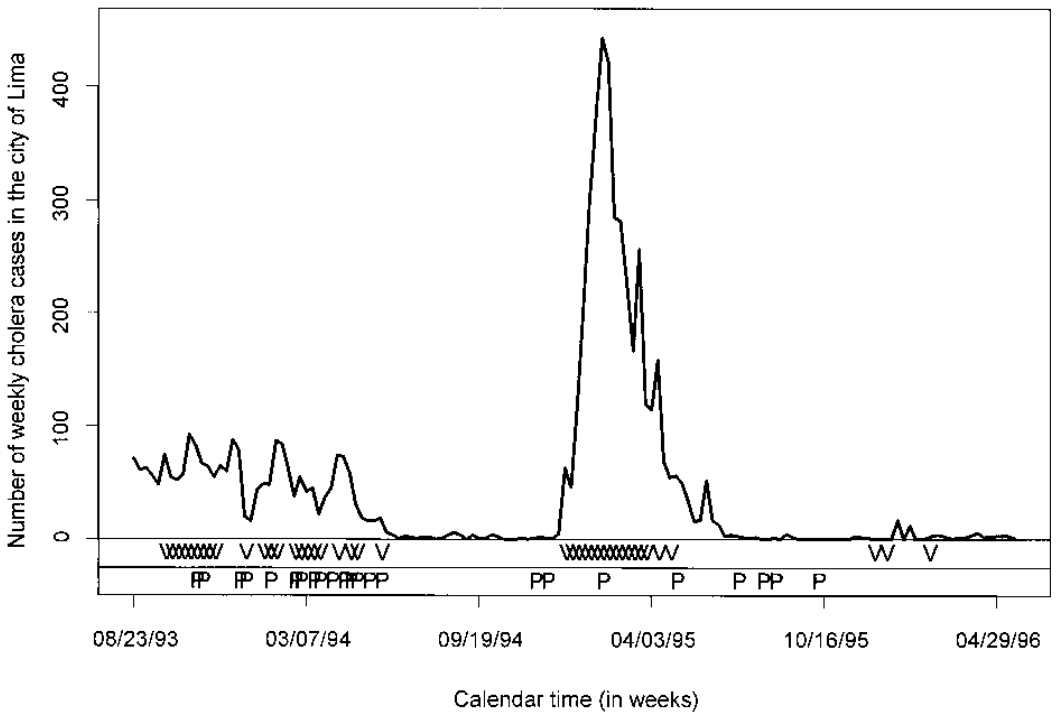

FIG. 1. Number of weekly cholera cases in Lima and the presence or absence of *V. cholerae* O1 and vibriophages in sewage water (August 1993 to May 1996). V, a positive isolation of *V. cholerae* O1 in sewage water; P, a positive isolation of vibriophages in sewage water. Dates are reported as month/day/year.

study, however, we used ambient temperature because of the difficulty of accessing sewage lines. Another community-based study of cholera conducted in Pampas de San Juan de Miraflores found a decrease in the number of cholera cases during an unseasonable cold spell (17a). In tropical countries, where ambient temperatures are nearly always above 20.0°C, factors other than temperature may influence the seasonality of cholera.

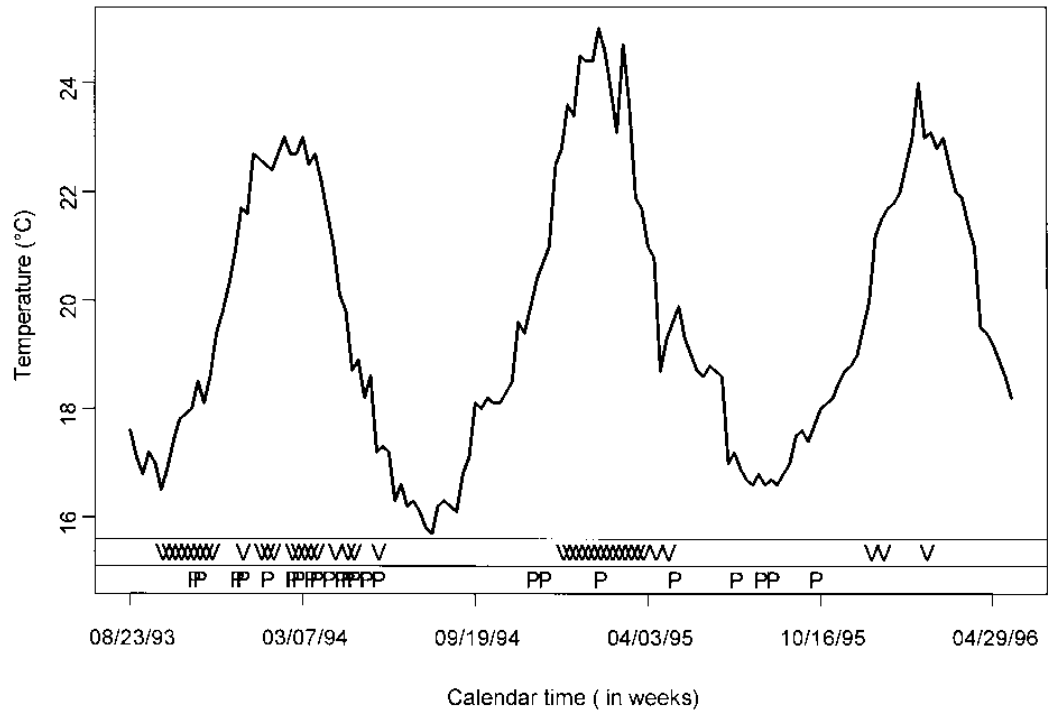

FIG. 2. Relationship between weekly ambient temperature and the presence of *V. cholerae* O1 and vibriophages in sewage water (August 1993 to May 1996). V, a positive isolation of *V. cholerae* O1 in sewage water; P, a positive isolation of vibriophages in sewage water. Dates are reported as month/day/year.

TABLE 1. Relationship between an outbreak of cholera in the city (i.e., five or more cases in a week) and *V. cholerae* O1 in sewage water

No. of cases/wk	Frequency of *V. cholerae* O1 isolations from sewage in the previous 4 wk[a]				
	None	+	++	+++	++++
<5	59	10	1	0	0
≥5	12	12	17	10	19
% of wk with ≥5 cases[b]	17	55	94	100	100

[a] For each week, we examined the number of positive *V. cholerae* O1 isolations in sewage water during the previous 4 weeks. Scale: +, at least one positive *V. cholerae* O1 isolation in the previous 4 weeks; ++++, all 4 weeks were positive for *V. cholerae* O1 in the previous 4 weeks.

[b] Weeks 1 through 4 of the study did not have data regarding the presence of *V. cholerae* O1 or vibriophages in sewage water during the four previous weeks, and for this reason we did not include these preliminary 4 weeks in the analysis. On the basis of our arbitrary definition of an outbreak, we classified the study weeks into one of the following: weeks with five or more cases and weeks with fewer than five cases.

In 1974, a cholera surveillance program in a South Africa mining community isolated *V. cholerae* O1 El Tor from sewage lines and from two healthy carriers 10 days before the report of the first clinical case of cholera (10). Isolation of cholera bacteria in sewage water depends on the presence of active cases and healthy carriers in the community (10) and also on ambient temperature in more temperate countries like Peru. The presence of *V. cholerae* O1 in sewage water was significantly associated with the occurrence of separate cholera outbreaks in the city of Lima, while the presence of vibriophages, although not statistically significant, was also related to the number of cholera cases in the city.

In the past, *Escherichia coli* phages have been used to detect non-enteropathogen-specific fecal contamination in water (7). To our knowledge, this study is the first to evaluate the presence of phages in sewage as a potential predictor of outbreaks of disease. This study uses simple and low-cost laboratory culture techniques (i.e., the cost of processing is under $5 per specimen), which can be implemented worldwide for detecting *V. cholerae* O1 and vibriophages in sewage. Further studies are needed to examine the utility of more sophisticated techniques such as PCR and indirect immunofluorescent antibody techniques for predicting cholera outbreaks by detecting *V. cholerae* O1 in the environment.

Vibriophages in sewage water appear to be related to the presence of *V. cholerae* O1 El Tor, since after purification they did not cross with *V. cholerae* non-O1 or other species of *Vibrio* nor with other enterobacteria. The vibriophages that we have isolated from sewage are hexagonal on electron microscopy (13a) and are different from the filamentous phage recently described as associated with cholera toxin production in *V. cholerae* O1 bacteria (21). No vibriophages in sewage were detected between March 1995 and May 1995 in spite of the high number of cholera cases in the city. Further investigation revealed that new vibriophage isolates were specific to the last *V. cholerae* O1 strains isolated in sewage but not to the LPS2 strain. To avoid this problem in the future, we recommend using both a strain of *V. cholerae* O1 such as MAK-757, which is a universal receptor for vibriophages (4), and the most recent *V. cholerae* O1 strain isolates from either infected patients or the environment.

TABLE 2. Relationship between an outbreak of cholera in the city (i.e., five or more cases in a week) and vibriophages in sewage water

No. of cases/wk	Frequency of vibriophage isolations from sewage in the previous 4 wk[a]				
	None	+	++	+++	++++
<5	51	16	3	0	0
≥5	29	19	15	7	0
% of weeks with ≥5 cases[b]	36	54	83	100	NA

[a] For each week, we examined the number of positive vibriophages isolations in sewage water during the four previous weeks. Scale: +, at least one positive vibriophage isolation in the previous 4 weeks; ++++, all 4 weeks were positive for vibriophages in the previous 4 weeks.

[b] Weeks 1 through 4 of the study did not have data regarding the presence of vibriophages in sewage water during the four previous weeks, and for this reason we did not include these preliminary 4 weeks in the analysis. On the basis of our arbitrary definition of an outbreak, we classified the study weeks into one of the following: weeks with five or more cases and weeks with fewer than five cases. NA, not applicable.

The role of vibriophages for monitoring cholera epidemics is unclear. Vibriophages are isolated from sewage more erratically than are *V. cholerae* O1 bacteria and appear to change their receptor depending on the *V. cholerae* O1 strain that is current. The one advantage that vibriophages may have is that in some years they were isolated from sewage prior to the appearance of *V. cholerae* O1, thus providing an earlier signal that a cholera outbreak may occur.

This study relied on the PMOH's reports of cholera cases for the city of Lima, which may be a limitation. The PMOH's clinical diagnosis is based on the presence of watery diarrheal disease accompanied by moderate to severe dehydration in individuals over the age of 5. Only one-third of the patients were sampled and cultured for *V. cholerae* O1, but 56% of the cultures sampled tested positive for the organism. The close relationship between confirmed and reported cases supports the sensitivity of the PMOH report criterion but does not indicate the number of cases which may not have been detected.

The signaling of a potential cholera outbreak several weeks in advance is a valuable tool for the implementation of various preventive measures in the community. It provides enough forewarning to increase the pressure and chlorination of water supplies, target health messages to communities at high risk, and mobilize resources to the community health centers to prepare for the treatment of greater numbers of cases. A longitudinal surveillance system for the detection of *V. cholerae* O1 and possibly vibriophages in sewage water at strategic points in populated cities offers an affordable monitoring system for cholera in less developed countries.

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