Diagnostic Limitations to Accurate Diagnosis of Cholera[▽]

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The treatment regimen for diarrhea depends greatly on correct diagnosis of its etiology. Recent diarrhea outbreaks in Bangladesh showed *Vibrio cholerae* to be the predominant cause, although more than 40% of the suspected cases failed to show cholera etiology by conventional culture methods (CMs). In the present study, suspected cholera stools collected from every 50th patient during an acute diarrheal outbreak were analyzed extensively using different microbiological and molecular tools to determine their etiology. Of 135 stools tested, 86 (64%) produced *V. cholerae* O1 by CMs, while 119 (88%) tested positive for *V. cholerae* O1 by rapid cholera dipstick (DS) assay; all but three samples positive for *V. cholerae* O1 by CMs were also positive for *V. cholerae* O1 by DS assay. Of 49 stools that lacked CM-based cholera etiology despite most being positive for *V. cholerae* O1 by DS assay, 25 (51%) had coccoid *V. cholerae* O1 cells as confirmed by direct fluorescent antibody (DFA) assay, 36 (73%) amplified primers for the genes *wbe* O1 and *ctxA* by multiplex-PCR (M-PCR), and 31 (63%) showed El Tor-specific lytic phage on plaque assay (PA). Each of these methods allowed the cholera etiology to be confirmed for 97% of the stool samples. The results suggest that suspected cholera stools that fail to show etiology by CMs during acute diarrhea outbreaks may be due to the inactivation of *V. cholerae* by *in vivo* vibriolytic action of the phage and/or nonculturability induced as a host response.

Cholera is a harsh disease, the fundamental clinical feature of which is severe dehydrating diarrhea that can lead to rapidly progressing dehydration and death. The recent cholera epidemics that occurred in South America (7), Asia (8), and sub-Saharan Africa (18) affected millions of people, with a high mortality rate. The World Health Organization (WHO) annual figures on global cholera incidence (26), which are based on official cases reported by affected countries, are believed to be underestimated due to limitations related to a lack of adequate surveillance systems. In addition, the actual number of cholera cases globally is estimated to be much higher than officially reported (22) because outbreaks are often not reported to avoid the risk of travel and trade embargoes on the affected country.

Prompt and accurate diagnosis of *Vibrio cholerae* is a key step in cholera outbreak surveillance that can greatly influence rapid intervention and prevention to minimize disease spread and mortality. Conventional culture methods (CMs) currently used for diagnosis of *V. cholerae* remain the gold standard, but this procedure is not precise and requires highly skilled technicians and laboratory infrastructure. In remote settings where cholera is endemic and modern laboratory facilities are often

nonexistent, simple dark-field microscopy to detect cells showing characteristic darting motility is used to identify *V. cholerae* in stool specimens. Diagnostic tests known as cholera dipstick (DS) assays, which involve either cholera toxin (3) or lipopolysaccharide (LPS) antigens (15, 21), have been introduced for rapid bedside detection of *V. cholerae* in cholera stools. DS tests produce results comparable to those from the CMs, perhaps because both require the physical presence of *V. cholerae* cells, although only readily culturable cells respond when CMs are used. Fluorescent monoclonal antibody (6) and PCR-based (13) methods have been proposed for detecting the cholera bacterium, including nonculturable cells present in stools.

In recent diarrheal outbreaks in Bangladesh, analysis of acute diarrhea cases showed *V. cholerae* to be the most commonly identified causative agent, even when approximately half of the stool samples from suspected cholera patients did not have any defined etiology (4). In the present study, stool samples collected from suspected cholera patients who had fallen ill during recent seasonal outbreaks in Bangladesh were subjected to extensive analyses by CMs, multiplex PCR (M-PCR), and direct fluorescent antibody (DFA) tests to determine etiology. The stool samples were also analyzed by plaque assay (PA) for the presence of vibriolytic phages in the stools.

MATERIALS AND METHODS

Collection of stool specimens. Suspected cholera stool specimens were collected from patients enrolled in the 2% systematic routine surveillance system at the Clinical Research and Service Centre of the International Centre for Diarrheal Disease Research, Bangladesh (ICDDRB). In this surveillance system,

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every 50th patient attending the hospital is screened for major enteric pathogens. The Dhaka hospital of the ICDDRB identifies cases of cholera throughout the year and treats patients during large outbreaks of the disease. According to the hospital surveillance records, about 88% of the patients report to the ICDDRB Dhaka hospital within <1 day after the first episode of acute diarrhea, while 12% report between 1 and 3 days. For this study, rice water stool samples were collected aseptically from 135 patients who had no prior medication, except that 88% had eaten rice gruel and the remaining 12% had been treated with rice oral rehydration saline (ORS) when they reported to the ICDDRB hospital in Dhaka between March and August 2009. The stool samples for culture (CMs) were immediately placed into Cary Blair medium (sodium thioglycolate, 1.5 g/liter; disodium phosphate, 1.1 g/liter; calcium chloride, 0.1 g/liter; sodium chloride, 5 g/liter; agar, 5 g/liter; pH 8.4) and transported to the laboratory within 30 min. Freshly collected stool samples were used for the dipstick assay, and aliquots were preserved at −20°C in glycerol for subsequent detection of *V. cholerae* by other methods.

Culture. About 5 to 10 μl of the stool specimen was streaked using an inoculating loop onto conventional taurocholate-tellurite-gelatin agar (TTGA) (Difco Laboratories, Detroit, MI) and incubated at 37°C for 18 to 24 h. Colonies with the characteristic appearance of *V. cholerae* (translucent with a black center) were subcultured on gelatin agar and confirmed by molecular methods as described previously (1, 10, 16).

Serogrouping. *V. cholerae* colonies from gelatin agar (GA) plates were tested to identify their serogroups using slide agglutination with polyvalent antiserum, followed by monoclonal and *V. cholerae* serogroup O1- and O139-specific antisera (1, 10). Samples that were negative for *V. cholerae* O1 or O139 by CMs were stored at −20°C and later examined by DFA assay and multiplex PCR, the latter for concurrent detection of *wbe* and *wbf* sequences specific for O1 and O139 serogroups of *V. cholerae*, respectively, and for *ctxA*-specific sequences (5, 16). Toxigenic *V. cholerae* O1 (strain MAK 757 El Tor) and O139 (strain AI1852) were used as positive controls for the multiplex PCR. The same stool samples were also tested for vibriolytic phage by plaque assay (12).

Dipstick assay. The dipstick test was performed by simultaneously introducing both O1 and O139 dipsticks into 200 μl of freshly collected stool sample contained in a tube (5). A positive result appeared as two pink lines (an upper control line and a lower LPS-positive line), and a negative result was a single upper pink control line. The results were discernible within 10 min. Dipsticks were developed to comprise a Crystal VC immunochromatographic one-step visual test for rapid detection of *V. cholerae* O1 and O139. The DS tests were provided by Span Diagnostics Ltd., Surat, India, and shipped by courier to the ICDDRB laboratory in ambient temperature (25 to 35°C).

DFA. Direct fluorescent antibody (DFA) detection of *V. cholerae* O1 was carried out following a method described elsewhere (1, 9, 17). Briefly, samples were preincubated in the dark overnight with 0.025% yeast extract (Difco Laboratories, Detroit, MI) and 0.002% nalidixic acid (Sigma). The samples were then centrifuged, and the pellet was stained using fluorescein isothiocyanate-labeled antiserum specific for O1 or O139 obtained from New Horizon Diagnostic Corp. (Columbia, MD). The stained samples were observed at ×1,000 magnification under UV light using an epifluorescence microscope (BX51; Olympus, Japan) connected to a digital camera (DP20; Olympus).

DNA preparation. Template DNA preparation was carried out according to a previously described method (16). Briefly, an aliquot (250 μl) of each stool sample was enriched in alkaline peptone water (APW) and heated in a boiling water bath for 10 min. For PCR assays, the stool samples were diluted using Tris-EDTA (pH 8.0) as the diluent. Bovine serum albumin (BSA) was added to the stool samples at a concentration of 400 ng μl^{-1} to circumvent the problem

of inhibitors of PCR present in stools. Samples were then centrifuged at 2,000 × *g* for 2 min to remove debris, and the supernatant was used as the template DNA.

M-PCR assays. M-PCR for amplification of primers for the *wbe* and *wbf* gene sequences, specific to the O1 and O139 serogroups of *V. cholerae*, respectively, was performed as described previously (16). The genes responsible for O-antigen biosynthesis and for generation of serotype-specific determinants are located in the *rfb* region on the *V. cholerae* chromosome. The *rfb* genes specific for *V. cholerae* O1 and O139 and the *ctxA* gene encoding subunit A of cholera toxin were amplified using M-PCR. Primer sequences for *V. cholerae* O1 *rfb*, *V. cholerae* O139 *rfb*, and *ctxA* are listed in Table 1. Amplification with the three primer pairs for genes (O1 *rfb*, O139 *rfb*, and *ctxA*) was performed simultaneously in 0.2-ml microcentrifuge tubes, and 3-μl samples were added to the PCR mixture to achieve a 30-μl final volume containing 0.21 mM each deoxynucleoside triphosphate (dNTP) mixture, 50 mM KCl, 1.5 mM MgCl$_2$, 10 mM Tris-HCl (pH 8.3), 0.17 μM *ctxA* primer pair, 0.27 μM (each) O1 and O139 *rfb* primer pairs, and 0.75 units of *Taq* polymerase (Takara, Kyoto, Japan). The conditions for amplification were 5 min at 94°C for initial denaturation of DNA and 35 cycles, each consisting of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, with a final round of extension for 7 min at 72°C in a DNA Robo Cycler Gradient Temperature Cycler (Strategene, La Jolla, CA). After amplification, 6 μl of each reaction mixture was subjected to electrophoresis on a 3% agarose gel (11 cm by 14 cm) using a horizontal electrophoresis apparatus (Horizon 11.14; Life Technologies, Gibco BRL). The gel containing amplified DNA was stained with ethidium bromide and visualized using a UV transilluminator. Images of the transilluminator were digitized using a one-dimensional Gel documentation system (Bio-Rad).

Phage assay. Two strains of *V. cholerae* O1 El Tor isolated from the same outbreak were used separately as hosts to determine the presence of vibriolytic phages by plaque assay as described previously (12). Briefly, 100 μl of logarithmic-phase cells of each *V. cholerae* O1 El Tor host strain in nutrient broth (Difco, Detroit, MI) was mixed with 3.9-ml aliquots of soft agar (Bactoagar; Difco), and the mixtures were overlaid on nutrient agar plates. The bacterium-free liquid part of the cholera stool, prepared by filtering the stool through 0.22-μm-pore-size filters (Millipore), was inoculated onto the seeded agar plates, and the plates were incubated for 16 h at 37°C. A sample was considered positive for vibriolytic phages when the lysed cells formed a plaque observed on the bacterial lawn.

RESULTS

Of 135 acute-diarrhea stool samples analyzed, 86 (64%) were positive for *V. cholerae* serogroup O1 by CMs, while 119 (88%) samples tested positive when the rapid cholera DS assay

TABLE 1. Targeted genes, primer sequences, and amplicon sizes[a]

Primer no.	Target gene (direction[b])	Primer sequence	Amplicon size (bp)
1	*ctxA* (F)	5′-CTCAGACGGGATTTGTTAGGCACG-3′	302
2	*ctxA* (R)	5′-TCTATCTCTGTAGCCCCTATTACG-3′	302
3	O1 *wbe* (F)	5′-GTTTCACTGAACAGATGGG-3′	192
4	O1 *wbe* (R)	5′-GGTCATCTGTAAGTACAAC-3′	192
5	O139 *wbf* (F)	5′-AGCCTCTTTATTACGGGTGG-3′	449
6	O139 *wbf* (R)	5′-GTCAAACCCGATCGTAAAGG-3′	449

[a] The PCR primers were designed following the method of Hoshino et al. (16).
[b] F, forward; R, reverse.

TABLE 2. Isolation and detection of the etiological agent of acute diarrhea in stool samples from patients suspected of having cholera

Result	Detection of *V. cholerae* by[a]:								Total no. of samples positive for *V. cholerae* O1[d]
	CM		DS		DFA		M-PCR	*V. cholerae* O1 El Tor-specific lytic phage on PA	
	O1	O139	O1	O139	O1	O139			
Culture positive	86	0	83	0	ND	ND	ND	ND	86
Culture negative	49	0	36	0	25 (22)	0	36 (30)[b]	31(26)[c]	45

[a] No. of samples in which *V. cholerae* was detected. ND, not done. The numbers in parentheses indicate the numbers that were dipstick positive.
[b] Four of the 36 M-PCR-positive stools were negative for *V. cholerae* O1 etiology by all other methods employed.
[c] Two of the 31 vibriolytic phage assay-positive stools were negative for *V. cholerae* O1 etiology by all other methods employed.
[d] Each of these methods confirmed *V. cholerae* O1 El Tor etiology for 97% of the acute diarrhea cases suspected to be from cholera.
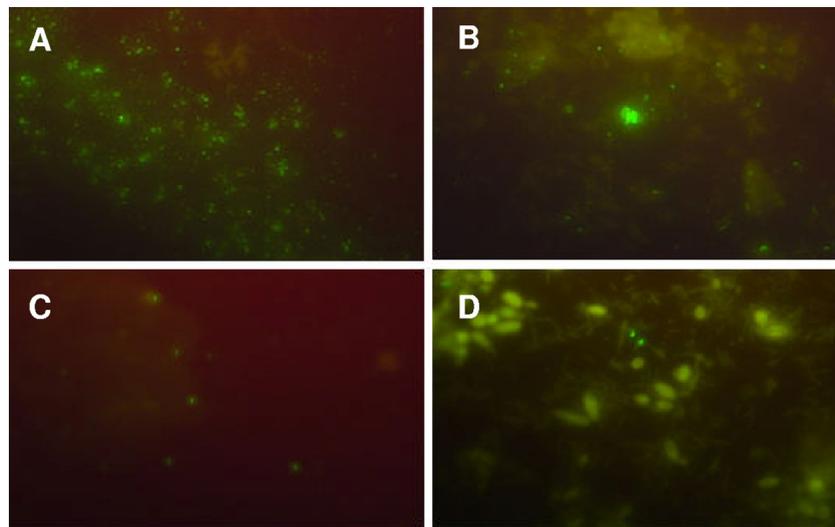
FIG. 1. DFA detection of nonculturable *V. cholerae* O1 in suspected cholera stool samples. (A to D) Stool samples were stained using a DFA reagent specific for *V. cholerae* O1 (New Horizon Diagnostic Inc., MD) and visualized using an epifluorescence microscope (Olympus model BX51, BX2 series) (6). The microscopic images were captured digitally (Olympus; DP 20) and processed using Adobe Photoshop version 5. Shown are *V. cholerae* O1 cells in stool samples that were culture positive (A), culture negative but DS positive (B), both culture and DS negative but M-PCR positive (C), and negative for *V. cholerae* O1 by culture, DS, and PCR but positive by DFA and vibriolytic plaque assay (D).

was used (Table 2). All but three samples positive for *V. cholerae* O1 by CMs also tested positive for *V. cholerae* O1 using the DS assay. Since the DS assay results suggested cholera etiology for a significant number of suspected stools that were negative for *V. cholerae* by CMs, all culture-negative stool samples preserved at −20°C were further examined using microbiological and molecular tools to confirm etiology within 2 weeks of sample collection. Of 49 culture-negative but suspected cholera stools, which included 36 (73%) samples that tested positive for *V. cholerae* using a specific DS assay, 25 (51%) showed the presence of coccoid *V. cholerae* O1 cells (Table 2) confirmed by the DFA method (Fig. 1). These results highlight the limitations of CMs in detecting etiology for a significant proportion of the infected cholera patients.

To complement the results obtained using DS and DFA, the samples were further analyzed by M-PCR for specific amplification of targeted genes and culture-independent detection of toxigenic *V. cholerae*. Of 49 template DNA samples tested, 36 (73%) amplified the primers for toxigenic *V. cholerae* O1-specific genes, *wbe* O1 and *ctxA* (Fig. 2), 30 of which (61%)

corresponded to DS-positive samples (Table 2). Four of the 36 M-PCR-positive stool samples tested negative for *V. cholerae* O1 etiology by all other methods employed. These results provided additional evidence for a cholera etiology, as indicated by DS and DFA, when CMs provided negative results for *V. cholerae*.

The culture-negative stool samples in the present study were further analyzed by PA to determine the presence of specific vibriolytic phages. Of 49 culture-negative but suspected cholera stools tested, 31 (63%) produced a clear lytic zone (Table 2) on a lawn of *V. cholerae* O1 El Tor that had been isolated from the same outbreak (Fig. 3). A major proportion of the culture-negative but DS-positive stool samples contained *V. cholerae* O1 El Tor-specific lytic phages, although the presence of vibriolytic phages was not indicative of whether the samples were positive for *V. cholerae* when M-PCR and/or DFA was used. Two of the 31 stools that tested positive for *V. cholerae* O1 El Tor-specific lytic phages by plaque assay were negative for cholera etiology by all other methods employed.

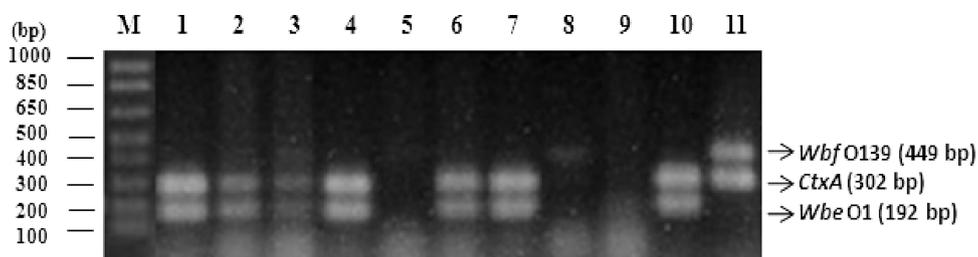When combined, each of these methods confirmed *V. chol-*

FIG. 2. Direct M-PCR detection of *V. cholerae* O1 *rfb* (*wbe*) and *ctxA* genes in template DNA preparations produced from suspected cholera stools that were culture negative for *V. cholerae*. Lanes 1 to 9 represent stool samples that supported the amplification of *V. cholerae wbe* O1 and *ctxA* in lanes 1 to 4, 6, and 7, but not in lanes 5, 8, and 9. Lanes 10 and 11 represent positive controls for *V. cholerae* O1 *rfb* (*wbe* O1) and *ctxA* genes and *V. cholerae* O139 *rfb* (*wbf* O139), respectively. Lane M, 1Kb Plus DNA Ladder (Invitrogen, Carlsbad, CA). The M-PCR-amplified product was subjected to agarose gel electrophoresis, and the DNA was visualized using ethidium bromide staining and UV transillumination.
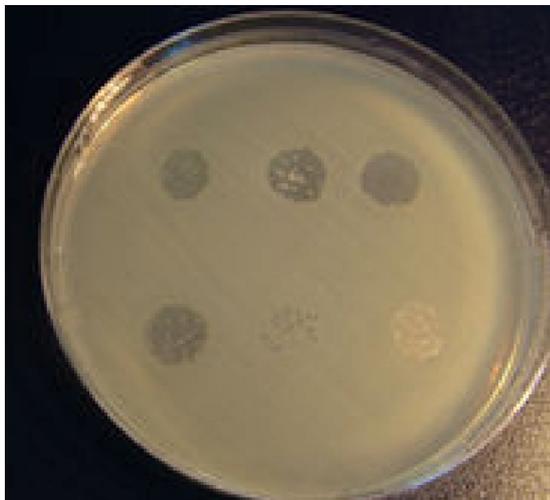
FIG. 3. Vibriolytic (*V. cholerae* serogroup O1 El Tor-specific) phage shown by PA. For the plaque assay, a bacterial lawn was prepared on Luria-Bertani (LB) agar using freshly grown *V. cholerae* O1 bacteria isolated from a cholera patient during an ongoing cholera outbreak. Suspected cholera stool samples from the same outbreak were diluted (10×) in LB broth, and a drop from each of the dilutions was placed on the bacterial lawn. The plates were incubated overnight at 37°C; the vibriolytic zone, in the form of PFU, was examined; and the result was digitized.

erae etiology for 97% of the acute diarrheal stools that had been suspected to be from cholera (Table 2).

DISCUSSION

Diarrhea is one of the leading causes of death in infants and children in many developing countries, including Bangladesh (2). Although the major causes of diarrhea include bacteria, viruses, and parasites (2, 20), cholera, caused by *V. cholerae*, is the most severe of all diarrheas and without treatment can kill half of affected individuals. Confirmed cases of cholera are routinely treated with a 1- to 3-day course of effective antibiotics (23, 24), along with rehydration therapy, to shorten illness and reduce both rapid water loss (19, 23) and the period of vibrio excretion. However, clinical management of cholera-like acute diarrhea is often jeopardized because the etiology is not defined for almost half of the cases (4). This study shows the practical limitations related to the precise diagnosis of cholera.

Supporting previously published data, diagnostic tests, such as the DS assay, used for rapid detection of *V. cholerae* antigens in stool samples were 24% more sensitive than CMs in the present study (7, 14, 21). Given that CMs are widely used for the purpose of isolation of *V. cholerae* and remain the standard for epidemiological surveillance of cholera, the greater sensitivity of rapid diagnostic kits compared with CMs invariably brings specificity into question, because sensitivity and specificity are the two key parameters for any rapid DS assay method that would be widely accepted and reliably used as an alternative to CMs. Half of the negative culture samples that were DS positive were also positive when tested for *wbe* O1 and *ctxA* using M-PCR, providing additional evidence that *V. cholerae* O1 was present. However, three stool samples testing negative for *V. cholerae* by DS assay, despite being culture

positive, contradicted the supposedly high sensitivity of the DS assay, as observed in the present study. This result may be explained by assuming that the culturable *V. cholerae* cells from the three stool samples positive by CMs likely had numbers of cells below the minimum required for the DS assay to produce a positive result.

Clinical cases of cholera that do not yield positive results using CMs may be influenced by factors such as changes in pH or osmolarity during passage through the lower alimentary tract or by antibiotics (25). *V. cholerae* responds rather quickly to such factors and transforms into a coccoid nonculturable cell that escapes diagnosis by CMs (9, 17). Coccoid *V. cholerae* O1 cells observed in cholera stools early in a cholera episode can significantly limit diagnosis if conventional CMs are used or if the characteristic darting motility of cells under dark-field microscopy is employed as a means to diagnose *V. cholerae* O1. This presents a serious diagnostic impediment that is related mainly to the nonculturability of *V. cholerae* (9) induced *in vivo* in response to host defenses (11, 12).

Recent studies in Bangladesh suggest that lytic bacteriophages specific for *V. cholerae* kill the bacteria present in the environment (12). It has been proposed that seasonal epidemics of cholera were controlled by the lytic action of *V. cholerae*-specific phages present in the environment (12). In the present study, more than half of the DS-positive but culture-negative stool samples contained *V. cholerae* O1 El Tor-specific lytic phages, suggesting cholera etiology. Although vibriolytic phages were detected irrespective of whether the samples were positive for *V. cholerae* by M-PCR and/or DFA, each of these methods confirmed *V. cholerae* etiology in nearly all of the acute diarrheal stools that had been suspected to be from cholera (Table 2). The phage assay was not done for stool samples that were positive for *V. cholerae* O1 by CMs. However, plaques (i.e., clear zones of bacterial growth inhibition) in *V. cholerae* O1 colonies caused by coinfecting vibriolytic phage were detected in 40% (*n* = 56) of the cholera stools cultured on TTGA and 13% of the stool samples lacking cholera etiology by CMs with specific phage when tested against *V. cholerae* isolated from the same recent outbreak in a follow-up study carried out by M. Alam, A. Sadique, Nur A. Hasan, G. Balakrish Nair, R. Bradley Sack, Anwar Huq, Rita R. Colwell, H. Watanabe, and Alejandro Cravioto (unpublished data). This result suggests a role for *in vivo* coinfecting phages in the elimination of susceptible *V. cholerae* bacteria from cholera stools, although host-mediated phage amplification was shown to occur in a separate compartment of the host lumen independent of the coinfecting *V. cholerae* causing the cholera epidemic. Thus, the decline of a cholera outbreak was hypothesized to be the result of increasing numbers of phage *from* the aquatic environment (12). However, the results presented in this study suggest that a significant proportion of suspected stools that do not test positive for *V. cholerae* by CMs most likely are due to phage elimination of *V. cholerae* from the guts of infected people.

WHO global incidence estimates for cholera (http://www.who.int/wer) show a significant (30%) increase in the number of cases between 2004 (101,383) and 2005 (131,943). Although the number of countries reporting cholera remained the same, a further 79% increase was shown between 2005 and 2006 (236,860 cases). Similarly, the worldwide cholera case fatality

rate also rose from 1.72% in 2005 to 2.66% in 2006. However, due to underreporting and other limitations of surveillance systems, WHO official figures for the global incidence of cholera are recognized as being incomplete. The results of the study reported here show that about 40% of suspected cholera cases occurring during acute diarrhea outbreaks and lacking a confirmed etiology (4) have *V. cholerae* as the predominant causative agent. Those cases that were erroneously confirmed as negative for *V. cholerae* O1 using CMs are also missed during cholera surveillances that determine the true global burden of the disease, which are reported in figures by the WHO.

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