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 diarrheal 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 dehydration 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 (MPCR), 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 (ICDDR,B). In this surveillance system,
every 90th patient attending the hospital is screened for major enteric pathogens. The Dhaka hospital of the ICDDR,B identifies cases of cholera throughout the year and treats patients during large outbreaks of the disease. According to the hospital surveillance reports, about 88% of the patients report to the ICDDR,B 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 ICDDR,B hospital in Dhaka between March and August 2009. The stool samples for culture (CMs) were immediately placed into Cary Blair medium (sodium thiglycolate, 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 monovalent and V. cholerae serogroup O1- and O139-specific antiserum (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). Toxicogenic 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 containing 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 ICDDR,B 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 Laboratory, 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 water bath for 10 min. For PCR assays, the stool samples were diluted using sample was enriched in alkaline peptone water (APW) and heated in a boiling

\[ \text{Table 1. Targeted genes, primer sequences, and amplicon sizes}^a \]

<table>
<thead>
<tr>
<th>Primer no.</th>
<th>Target gene (direction)</th>
<th>Primer sequence</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ctxA (F)</td>
<td>5'-CTCAAGGACCGAATTGTGAAGGACG-3'</td>
<td>302</td>
</tr>
<tr>
<td>2</td>
<td>ctxA (R)</td>
<td>5'-TCTAATAGGCAGCGCGCTAGC-3'</td>
<td>302</td>
</tr>
<tr>
<td>3</td>
<td>O1 wbe (F)</td>
<td>5'-GTTAGCAAGCAAGTGGGGG-3'</td>
<td>192</td>
</tr>
<tr>
<td>4</td>
<td>O1 wbe (R)</td>
<td>5'-GGTAGCTGATGAGAACAG-3'</td>
<td>192</td>
</tr>
<tr>
<td>5</td>
<td>O139 wbf (F)</td>
<td>5'-AGCTCTTATTACAGGTTG-3'</td>
<td>449</td>
</tr>
<tr>
<td>6</td>
<td>O139 wbf (R)</td>
<td>5'-GTCAACGGGATGTGAAAGG-3'</td>
<td>449</td>
</tr>
</tbody>
</table>

\[ a \text{ The PCR primers were designed following the method of Hoshino et al. (16).} \]

\[ b \text{ F, forward; R, reverse.} \]

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 ctxA, V. cholerae O139 ctxA, 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 MgCl₂, 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, Tokyo, 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 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

\[ \text{Table 2. Isolation and detection of the etiological agent of acute diarrhea in stool samples from patients suspected of having cholera}^a \]

<table>
<thead>
<tr>
<th>Result</th>
<th>CM</th>
<th>DS</th>
<th>DFA</th>
<th>M-PCR</th>
<th>V. cholerae O1 El Tor-specific lytic phage on PA</th>
<th>Total no. of samples positive for V. cholerae O1$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture positive</td>
<td>86</td>
<td>0</td>
<td>83</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Culture negative</td>
<td>49</td>
<td>0</td>
<td>36</td>
<td>0</td>
<td>25 (22)</td>
<td>0</td>
</tr>
</tbody>
</table>

\[ a \text{ No of samples in which V. cholerae was detected. ND, not done. The numbers in parentheses indicate the numbers that were dipstick positive.} \]

\[ b \text{ Four of the 36 M-PCR-positive stools were negative for V. cholerae O1 etiology by all other methods employed.} \]

\[ c \text{ Two of the 31 vibriolytic phage assay-positive stools were negative for V. cholerae O1 etiology by all other methods employed.} \]

\[ d \text{ Each of these methods confirmed V. cholerae O1 El Tor etiology for 97% of the acute diarrhea cases suspected to be from cholera.} \]
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-
pared on Luria-Bertani (LB) agar using freshly grown *V. cholerae* phage shown by PA. For the plaque assay, a bacterial lawn was pre-
at 37°C; the vibriolytic zone, in the form of PFU, was examined; and the plates were incubated overnight
was placed on the bacterial lawn. The plates were incubated overnight
was 56) of the cholera stools cultured
were diluted (10×) in LB broth, and a drop from each of the dilutions
et zones were 24% more sensitive than CMs in the
were detected in 40% (*n* = 56) of the cholera stools cultured on TTGA and 13% of the stool samples lacking cholera etiol-
recent epidemic. Thus, the decline of a cholera outbreak was hypoth-
rics of cholera were controlled by the lytic action of *V. cholerae-
specific phages present in the environment (12). In the present
was not done for stool samples that were positive for *V. cholerae* O1 by CMs. However,
plaque 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
was 79% increase was shown between 2005 and 2006
on TTGA and 13% of the stool samples lacking cholera etiology in nearly all of the
to the nonculturability of *V. cholerae* (9) induced in vivo in response to host defenses (11, 12).
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). Cocoid *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 bacterio-
plagues (i.e., clear zones of bacterial growth inhibition) in
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
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,
plagues for 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


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

\[\text{erae etiology for 97% of the acute diarrheal stools that had been suspected to be from cholera (Table 2).} \]

\[\text{DISCUSSION} \]

\[\text{Diarrhea is one of the leading causes of death in infants and children in many developing countries, including Bangladesh (2). Although the major causes of diarrhea include bacteria, viruses, and parasites (2, 20), cholera, caused by } \]

\[\text{V. cholerae, is the most severe of all diarrheas and without treatment can kill half of affected individuals. Confirmed cases of cholera are routinely treated with a 1- to 3-day course of effective antibiotics (23, 24), along with rehydration therapy, to shorten illness and reduce both rapid water loss (19, 23) and the period of vibrio excretion. However, clinical management of cholera-like acute diarrhea is often jeopardized because the etiology is not defined for almost half of the cases (4). This study shows the practical limitations related to the precise diagnosis of cholera.} \]

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\[\text{by DS assay, despite being culture positive, contradicted the supposedly high sensitivity of the DS assay, as observed in the present study. This result may be explained by assuming that the culturable } \]

\[\text{V. cholerae cells from the three stool samples positive by CMs likely had numbers of cells below the minimum required for the DS assay to produce a positive result.} \]

\[\text{Clinical cases of cholera that do not yield positive results using CMs may be influenced by factors such as changes in pH or osmolarity during passage through the lower alimentary tract or by antibiotics (25). } \]

\[\text{V. cholerae responds rather quickly to such factors and transforms into a coccoid nonculturable cell that escapes diagnosis by CMs (9, 17). Cocoid } \]

\[\text{V. cholerae O1 cells observed in cholera stools early in a cholera episode can significantly limit diagnosis if conventional CMs are used or if the characteristic darting motility of cells under dark-field microscopy is employed as a means to diagnose } \]

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\[\text{V. cholerae (9) induced in vivo in response to host defenses (11, 12).} \]

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phages specific for } \]

\[\text{V. cholerae} \]

\[\text{kill the bacteria present in the environment (12). It has been proposed that seasonal epidem-
ics of cholera were controlled by the lytic action of } \]

\[\text{V. cholerae-specific phages present in the environment (12). In the present study, more than half of the DS-positive but culture-negative stool samples contained } \]

\[\text{V. cholerae O1 El Tor-specific lytic phages, suggesting cholera etiology. Although vibriolytic phages were detected irrespective of whether the samples were positive for } \]

\[\text{V. cholerae by M-PCR and/or DFA, each of these methods confirmed } \]

\[\text{V. cholerae etiology in nearly all of the} \]

\[\text{acute diarrheal stools that had been suspected to be from cholera (Table 2). The phage assay was not done for stool samples that were positive for } \]

\[\text{V. cholerae O1 by CMs. However, plaques (i.e., clear zones of bacterial growth inhibition) in } \]

\[\text{V. cholerae O1 colonies caused by coinfecting vibriolytic phage were detected in 40% (} \]

\[\text{n = 56) of the cholera stools cultured on TTGA and 13% of the stool samples lacking cholera etiology by CMs with specific phage when tested against } \]

\[\text{V. cholerae isolated from the same recent outbreak in a follow-up study carried out by M. Alam, A. Sadique, Nur A. Hasan, G. Bal-
akrish Nair, R. Bradley Sack, Anwar Huq, Rita R. Colwell, H. Watanabe, and Alejandro Cravioto (unpublished data). This result suggests a role for in vivo coinfecting phages in the elimination of susceptible } \]

\[\text{V. cholerae bacteria from cholera stools, although host-mediated phage amplification was shown to occur in a separate compartment of the host lumen inde-
pendent of the coinfecting } \]

\[\text{V. cholerae causing the cholera epidemic. Thus, the decline of a cholera outbreak was hypo-
thesized to be the result of increasing numbers of phage from the aquatic environment (12). However, the results presented in this study suggest that a significant proportion of suspected stools that do not test positive for } \]

\[\text{V. cholerae by CMs most likely are due to phage elimination of } \]

\[\text{V. cholerae from the guts of infected people.} \]

\[\text{WHO global incidence estimates for cholera (http://www.who.int/wer) show a significant (30%) increase in the number of cases between 2004 (101,383) and 2005 (131,943). Although the number of countries reporting cholera remained the same, a further 79% increase was shown between 2005 and 2006 (236,860 cases). Similarly, the worldwide cholera case fatality} \]
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 surveillance that determine the true global burden of the disease, which are reported in figures by the WHO.

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