Original Article

Evaluation of a Rapid Immunochromatographic Dipstick Kit for Diagnosis of Cholera Emphasizes Its Outbreak Utility

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SUMMARY: We evaluated the Crystal VC®, a commercially produced dipstick, for the rapid detection of *Vibrio cholerae* serotypes O1 and O139 directly from the stool samples of hospitalized diarrheal patients using the conventional bacteriological method as gold standard. The sensitivity and specificity of the dipsticks were about 92 and 73%, respectively. Introduction of the PCR-based method along with the classical bacteriological method as the gold standard for the evaluation of a kit may improve the sensitivity and specificity of the assay. The dipstick method requires minimal technical skill, and the test can be read in about 10 min. This dipstick test has the potential to act as an early warning system for cholera in many developing countries, especially during the start of an outbreak, which would ultimately lead to a decrease in the spread of the disease as well as the case fatality rate. Furthermore, the use of a rapid detection test will improve surveillance and thus reduce the burden of disease estimates, especially in remote settings.

INTRODUCTION

The epidemiology of cholera continues to be perplexing and several extraordinary events have been recorded in the recent past (1–5). In recent years, cholera has become endemic in several geographical areas, reflecting poor maintenance of hygiene related infrastructure and difficulties in implementation of control measures in developing countries. Nearly 120 countries have reported indigenous cases of cholera since 1991. The recent cholera statistics released by World Health Organization (WHO) paint a gloomy picture with both the number of cases and the number of countries reporting cases to WHO increasing during 2008, reaching the level of the late 1990s (6). The overall number of reported cases of cholera to WHO during the most recent 5 year period (2004–2008) showed a 24% increase from the number of cases between 2000 and 2004 (6). Globally, there was an increase in the number of deaths by 27.5%, representing an overall case fatality rate (CFR) of 2.7% (6). Several large outbreaks occurred in different parts of India during the last few years. Therefore, cholera continues to be a major problem in many developing countries.

Cholera surveillance remains an important component for determining trends of cholera incidence in different regions of the world as well as within individual countries. The officially reported figures to WHO on global incidence of cholera (6) underestimate the actual figures several fold, since cholera is underreported due to fear of unjustified travel and trade restrictions, and limitations in the surveillance system (7). Among the enteric pathogens, *Vibrio cholerae* is perhaps the easiest to identify, but this requires a laboratory infrastructure and skilled laboratory personnel. Cholera is a disease of the poor, and outbreaks and epidemics of cholera usually occur in peripheral or war-ravaged areas where laboratory facilities are unavailable or are grossly inadequate. Consequently, efforts have been made to develop simple rapid diagnostic tests (RDT), which would allow the diagnosis of *V. cholerae* in the hospital/field. The availability of a reliable RDT with high sensitivity and specificity will allow the detection of outbreaks early so that an effective response can be implemented in a timely manner. This will ultimately lead to a decrease in the spread of the disease as well as the CFR. Furthermore, the use of RDT will improve surveillance and thus reduce the burden of disease estimates.

Several RDT based on monoclonal antibodies against *V. cholerae* O1 or O139 have been evaluated (8–11). Though the sensitivity and specificity of these tests exceeded 95%, these assays are more complicated than the dipstick test and may not be suitable for use in the field (8,10). Researchers at the Institute Pasteur, Paris, France, recently developed a one-step immunochromatographic dipstick test for rapid detection of *V. cholerae* O1 and O139 from stool samples. In an evaluation of the dipstick assays in Madagascar and Bangladesh, two areas where cholera is endemic, the specificity of the O1 and O139 dipsticks ranged between 84 and 100% and their sensitivity ranged from 94.2 to 100% (12). When evaluated using rectal swabs from diarrheal patients incubated for 4 h in alkaline peptone water (APW), the sensitivity and specificity were greater...
than 92 and 91%, respectively (13). More recently, the prototype dipstick was evaluated under actual field conditions in a prospective study in Beira, Mozambique, during the 2004 cholera season (14). The dipstick method requires minimal technical skill, and the test can be read in about 10 min. In addition, the dipsticks can be stored at room temperature in a humidity-proof plastic bag, making them easily transportable (12).

Recently, the Institute Pasteur has transferred this technology to an India-based company (Span Diagnostics), where it is now being produced commercially. If the dipstick is to be used in actual field settings and recommended for detection of cholera cases, the performance of the commercially produced RDT needs to be evaluated in the laboratory using bulk stool (15). More recently, during a cholera epidemic in Guinea Bissau, epidemiologists from the US Centers for Disease Control and Prevention (CDC) and the Ministry of Health of Brazil, assisted by hospital workers from the municipal hospital in Bissau, evaluated the performance of the Crystal VC® Rapid Dipstick (VC) test, where the isolation rate of cholera was more than 73% and used PCR, rather than culture, as the diagnostic gold standard for all samples (16). This has prompted us to conduct a hospital-based evaluation of this new dipstick test in an endemic zone of cholera in Kolkata, where prevalence of V. cholerae ranges between 25 and 35%, to understand the efficacy of the dipsticks for the rapid detection of cholera directly from stool samples and its comparison with culture methods.

**MATERIALS AND METHODS**

**Study site and subjects:** Kolkata is the third largest city in India. It has a population of approximately 13 million, divided among 141 wards and it is one of the world’s most densely populated cities. Cholera is endemic in the city and surrounding areas, usually occurring from March to November each year. The Infectious Disease Hospital (IDH) in Kolkata is the main referral center for diarrhea in West Bengal. Diarrhea was defined as three or more loose bowel movements during a 24-h period. Cholera was defined as a diarrheal infection where V. cholerae O1/O139 was isolated.

There is an on-going etiologic surveillance of diarrheal diseases at the IDH by the National Institute of Cholera and Enteric Diseases (NICED) to which this study was appended. This institute serves as the National Reference Center as WHO collaborating organization. Patients of all age groups presenting to the IDH with acute, non-bloody, watery diarrhea were included into this study. Stool samples were obtained by rectal catheter from each consenting patient. Data on age, sex, severity of diarrhea and dehydration, and treatment received was extracted from the case report form.

**Dipstick test:** Crystal VC® (Span Diagnostics, Surat, India) is a lateral flow immunochromatographic test for the qualitative determination of lipopolysaccharide (LPS) antigen of both V. cholerae O1 and O139 serogroups from stool specimens, using monoclonal antibodies specific to V. cholerae O1 and O139 LPS (product literature). Each kit contains: (i) a dipstick individually packed in an aluminum pouch, (ii) a test tube for the stool sample, (iii) a disposable plastic dropper for transferring stool into the test tube; and (iv) buffer for diluting the stool samples.

During standardization of this dipstick test, initially the whole stool sample was used directly for performing the test and many false-positive cases were recorded, forcing a change to the protocol. During this standardization process, a representative from Span Diagnostics also worked in our laboratory. After several rounds of trials, a buffer solution was formulated and introduced to dilute the stool sample.

The test tube was filled with one drop (50 μL) of liquid stool and three drops of supplied buffer. The dipstick was placed in the test tube, such that the end of the dipstick marked with an arrow sign remained submerged in the liquid. The result was read within 10–15 min and the tests were defined as positive when both a test line for either O1 or O139, and the control line appeared on the test strip (Fig. 1).

**Conventional bacteriological culture:** Conventional bacteriological culture was applied as the gold standard against which the accuracy of the rapid test was evaluated. The specimens were plated directly onto thiosulfate citrate bile salt sucrose (TCBS) agar (Eiken Chemical, Tokyo, Japan). The specimens were also inoculated and incubated in APW (pH 8.6) for 6 to 8 h at 37°C then plated onto TCBS. After overnight incubation at 37°C, suspected colonies on the TCBS plates were tested biochemically and confirmed by agglutination with polyclonal O1 and monovalent Ogawa and Inaba antisera (Difco Laboratories, Detroit, Mich., USA). Nonagglutinating strains were tested with antisera to V. cholerae O139 strain. If the strain agglutinated with O1 or O139 antisera, the corresponding strain was considered as O1 or O139 V. cholerae, respectively.

![Fig. 1. Dipsticks showing typical negative and positive results after being kept for 5–10 min in secretory diarrheal stool sample and/or culture along with the supplied buffer. (A) Appearance of control band only indicating the sample is negative for both V. cholerae O1 and O139. (B) Appearance of two bands, upper one is control and the other is specific to O1 V. cholerae. (C) Appearance of two bands, upper one is control and the other is specific to O139 V. cholerae. (D) Appearance of three bands indicating presence of both V. cholerae O139 and O1 along with the control line.](image-url)
Sensitivity assay of the dipstick test: A suspension containing \(2 \times 10^5\) cells/mL was prepared in sterile phosphate buffer saline (PBS) from overnight grown archived \(V.\) cholerae O1, O139 and non-O1 non-O139 strains (5 strains for each category) isolated from patients with diarrhea and from this suspension 10-fold serial dilutions were made. From each dilution, 100 \(\mu L\) was spread on Luria agar plates and incubated overnight at \(37^\circ C\) for colony count. Another 200-\(\mu L\) aliquot from each dilution was used for the dipstick assay.

Multiplex PCR assay: The genes responsible for O-antigen biosynthesis and for the generation of serotype-specific determinants are located in the \(rfb\) region on the \(V.\) cholerae chromosome. The \(ompW\) gene, specific for \(V.\) cholerae and the \(rfb\), specific for \(V.\) cholerae O1 (O1-\(rfb\), were amplified using multiplex PCR assay. \(V.\) cholerae O1-\(rfb\) specific primers were 5'-GTTCATGGAAGTGTCAAAC-3' sense strand, and 5'-GGAAC TTATAACCACCGCG-3' antisense strand, while \(ompW\) specific primers were 5'-CACAAAGAAG GTGACCTTTTGTGG-3' sense strand, and 5'-GAAC TTATAACCACCGCG-3' antisense strand. Boiled APW enriched samples were used as DNA template. PCR was performed with a PTC 200 Peltier thermal cycler (M.J. Research, Watertown, Mass., USA). The following reagents were added to each sample for PCR mixture in a 200-\(\mu L\) PCR tube containing a reaction mixture volume of 25 \(\mu L\). Each reaction contained 2.5 \(\mu L\) of 10 \(\times\) PCR buffer (Takara, Otsu, Japan), 2.5 \(\mu L\) of 2.5 mM dNTP mix, 1 \(\mu L\) each of the forward and the reverse primer of \(ompW\) (10 pmol \(\mu L^{-1}\)), 1.5 \(\mu L\) each of the O1-\(rfb\) primer pairs (10 pmol \(\mu L^{-1}\)), 0.2 \(\mu L\) of amplitaq DNA polymerase (Takara) and 2.5 \(\mu L\) of DNA template and Milli-Q water to a final volume of 25 \(\mu L\). The PTC-200 instrument was programmed as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles consisting of 94°C for 1.5 min, 55°C for 1.5 min and 72°C for 1.5 min, and a final extension step at 72°C for 10 min at the end of 35 cycles, followed by maintenance at 4°C. \(V.\) cholerae O1 strain, N16961 was used as a positive control. Amplified product was separated on a 1.5% agarose gel and visualized with a gel documentation system (Bio-Rad, Hercules, Calif., USA).

RESULTS AND DISCUSSION

Among the 212 patients included in the study between July and December 2008, 72 were culture positive for \(V.\) cholerae O1 and 140 were negative for \(V.\) cholerae O1 culture. Among the 72 culture-positive cases, 66 were positive by the dipstick assay but 6 which were positive by culture were negative by the dipstick assay. By the dipstick assay, 104 cases indicated positive for \(V.\) cholerae O1. Thirty-eight cases, which indicated positive by the dipstick assay, were negative by direct culture as well as by APW enrichment culture. Overall, the sensitivity (SN), specificity (SP), positive-predictive value (PPV) and negative-predictive value (NPV) were 91.7, 72.9, 63.5, and 94.4%, respectively. It was found that during the monsoon, the results for sensitivity and specificity were much higher than the winter season which may be due to the higher isolation rate of \(V.\) cholerae O1 during the monsoon period. During the monsoon season (when the number of \(V.\) cholerae positive cases were more), for the 85 samples that were evaluated, the SN, SP, PPV, and NPV was 100, 87.3, 73.3, and 100%, respectively, whereas, during the post-monsoon and winter season (when non-\(V.\) cholerae cases were more prevalent), for the 127 samples that were evaluated, the SN, SP, PPV, and NPV was 88, 61, 59.5, and 88.7%, respectively. Although there were significant differences (\(P < 0.05\)) in the SN and SP between monsoon and post-monsoon season, to draw any final conclusion this kind of evaluation should be continued at least for 1 more year. We also compared the results between patients who required intravenous (i.v.) rehydration therapy during treatment versus patients who did not receive i.v. fluid. The results did not show any significant differences between these two groups.

During the study period, two non-O1 non-O139 positive-stool samples indicated negative by dipstick analysis. During the study period, 7 dipstick positive-stool samples, which were negative by direct plating on TCBS, indicated \(V.\) cholerae O1 positive after APW enrichment plating. There was no isolation of \(V.\) cholerae O139 strain during the study period and dipstick test did not produce any positive results among the 212 cases analyzed either. Twenty archived strains of \(V.\) cholerae O139 indicated a positive band in the expected position, which is different from the O1 band, when they were tested with the dipstick. Of 212 tests, not a single test lacked control lines as reported earlier by others (14).

When whole stool samples were used many false-positive cases were encountered, thus a dilution step was needed for this kit. It suggests that unknown substance(s) in human stool may nonspecifically interact with \(V.\) cholerae O1 antibody, possibly causing these false positives. This is consistent with the data of the Wang et al. report (14). Their data showed that the bulk stool group showed a lower specificity than the enriched rectal swab group. The enrichment in APW not only dilutes the unknown substances that may react with O1/O139 antibody (enhancing the specificity) but also increases the density of \(V.\) cholerae (potentially improving the sensitivity of the test). But APW enrichment needs 6-8 h incubation, whereas dilution needs no extra time. In the field situation, where laboratory facilities are miles away, inoculation in APW and incubation is hard to perform. So, use of dilution buffer helps to provide a result within 20 min.

The sensitivity assay of the RDT was verified using bacterial culture grown in liquid media. The specificity of the dipsticks was 100% for all bacterial cultures. When tested for sensitivity, the O1 and O139 line of the dipsticks was positive with all strains of \(V.\) cholerae O1 (100%) and O139 (100%), respectively. Non-O1 non-O139 strains did not yield any band except the control line. A minimum of ca. \(10^6\) CFU of \(V.\) cholerae O1/mL or \(10^5\) CFU of \(V.\) cholerae O139/mL is required to give an unequivocal positive reaction. This result is different from the Nato et al. (12). This may be due to the fact that the earlier group used the kit produced by the Institute Pasteur, which prepared the kit for O1 and O139 separately. But here we used the combo kit for O1 and O139, manufactured by Span Diagnostics that may have compromised the level of sensitivity for accommodating both the serogroups in a single kit. The figure also clear-
ly supports our finding that pure culture of O139 produced a less intense band as compared to the O1 positive-stool sample.

In this evaluation, the VC test showed moderately good sensitivity but lower specificity (Table 1). It must also be taken into consideration that the specificity results may also be underestimated as it is likely that sometimes the culture method may underestimate the number of true positive cases of cholera. The VC test can be recommended for the early detection of cholera epidemics caused by _V. cholerae_ O1 and O139 in order to accelerate the implementation of public health measures. In addition, the dipsticks can be stored at room temperature in a humidity-proof plastic bag, making them easily transportable.

In some cases, positive lines in the RDT were faint perhaps due to the low number of organisms in a single drop of sample, as evidenced by the result that some samples were positive only by enrichment culture (7 cases). We assume that interpretations of such results are subjective and require experience in judging the results. Hence, despite the apparent simplicity of the test, we recommend a short training/demonstration for field users of the kit.

Although conventionally the culture-based detection system seems to be the gold standard, sometimes culturing in selective media fails to detect the organism when the number of organisms is low in the sample or due to prior treatment with antibiotics taken from a local pharmacy “over the counter,” which is common practice in developing country settings. If the kit-based detection system gives a positive result for such a sample (LPS from dead cells), then interpretation regarding specificity of the kit will be wrong. On the other hand, the PCR-based detection system can yield a positive result even if the number of organisms is low or the cells are dead. We further analyzed 10 samples that were O1 dipstick positive but culture negative by the multiplex PCR assay (ompW and O1-rgb) using boiled APW enrichment samples as a template, and the results showed the presence of _V. cholerae_ O1 in 4 culture negative but RDT-positive samples. So, addition of the PCR-based detection system along with the culture method as the gold standard will help us to evaluate RDT in a meaningful way. Recently, one field study conducted by Harris et al. (16) in Guinea Bissau during an outbreak using PCR as the diagnostic gold standard found that the RDT kit was 97% sensitive and 71–76% specific, which is in accordance with our hospital-based findings.

Finally, we are of the same opinion as Harris et al. (16) that at this stage the VC kit cannot be used as definitive confirmation of _V. cholerae_ infection, particularly on a patient-by-patient basis. Instead, the utility of this test kit can be realized in a much better fashion during a suspected cholera outbreak, where several patients are tested simultaneously. A predominance of positive test results in this situation would strongly support _V. cholerae_ as the causative etiologic agent of the outbreak. However, a careful approach should be taken even when this occurs and attempts should always be made to ensure that at least some cases yield culture positive by conventional bacteriological method to validate the kit-based results. This will also allow antimicrobial susceptibility testing to be performed that may facilitate the formulation of the local treatment guidelines. In other words, this diagnostic testing may not be necessary for the clinical management of each diarrhea patient during an outbreak, but this dipstick test has the potential to act as an early warning system for cholera in many developing countries, especially during the start of an outbreak. Once a cholera outbreak has been confirmed, large-scale rapid preventive measures, including mass vaccinations, promotion of protective behavior related to food and water handling, improvement of hygiene and sanitation, and ensuring a sufficient supply of materials for cholera treatment, such as oral rehydration salts and antibiotics, can be mobilized to minimize morbidity and mortality so that an outbreak cannot progress into an epidemic. So, at this juncture we classify this kit as an “epidemiological test kit.” The introduction of the dipsticks may be most useful in remotely affected regions including refugee camps or war-ravaged areas where no alternative diagnostic test exists, because the test is simple and does not require any special infrastructure or laboratory facility.

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**REFERENCES**