STOP CHOLERA

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Manual for Detecting *Vibrio cholerae* O1 from Fecal Samples Using an Enriched Dipstick Assay – a Low-Cost, Simplified Method of Confirming Cholera

Updated November, 2016

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From DOVE Project www.stopcholera.org

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August 13, 2015

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A Note About This Document

This is a "working paper" that describes the use of a dipstick test to detect *Vibrio cholerae* O1 from fecal samples. Since it is a working paper, we expect that improvements and modifications will be made in the future. However, we believe that the methods described here will be useful for programs that need to confirm cholera cases. The DOVE Project and the authors welcome your suggestions for improving the methods or instructions on how to carry out these assays. The manual describes the use of Crystal VC because most field studies have used this product. The DOVE Project will update the manual to include other rapid tests as more experience is gained in their use.

Contents

Introduction: The importance of cholera surveillance and the need for improved laboratory testing methods in the field

Surveillance plays a critical role in the effective control of cholera. It is needed to detect cases at an early phase of an outbreak and to document the course of the infection in time and space. High-quality surveillance depends both on the ability to detect suspected cases of cholera and to confirm cases in the laboratory. The surveillance system should be able to collect, analyze and disseminate data in order to facilitate epidemiological forecasts and prepare for cholera control measures. However, establishing a high-quality disease surveillance system is not simple and can be both expensive and difficult, especially in remote areas of developing countries where cholera often occurs. Surveillance is especially challenging following a natural disaster or civil unrest when other priorities compete for attention.

A surveillance system includes both an epidemiological strategy and a laboratory component to confirm cases. The standard laboratory method for confirming cases is fecal culture of a representative sample of cases. To perform cultures, fecal specimens are generally collected using Cary-Blair transport medium and sent to a well-equipped reference laboratory with trained technicians for processing.[1] The laboratory methods for identifying *Vibrio cholerae* in a microbiology laboratory are well described in other manuals and will not be described here.[2] These methods have been used effectively during outbreaks, as well as in sentinel surveillance systems. However, in addition to the costs involved, there are constraints in relying solely on standard microbiology laboratory methods. Transport medium may not be available at the local hospital, and transporting to the reference laboratory and subsequently sending the reports back to the treating hospital takes time. There is often a one or even two-week interval between the time the fecal specimen is obtained and the report is received from the laboratory.

Complementary methods are now being used to supplement, and in some cases, replace standard microbiology. These include rapid diagnostic tests, which can be used at the local health facility, and polymerase chain reaction (PCR) tests to identify the DNA from *V. cholerae* in fecal specimens. The PCR methods are useful and may become the gold standard method, but at present, they are still primarily used for research purposes and are not readily adaptable for clinical use. This may change in the future as PCR becomes more widely available. In this manual, we describe a low-cost, simple method to confirm cholera cases using a cholera dipstick rapid test to detect *V. cholerae* O1 from fecal specimens of suspected cholera patients.

The objectives of laboratory surveillance

Laboratory detection and confirmation of cholera cases may be needed for one or more objectives. These include the following:

- Surveillance for early identification of cholera outbreaks
- Monitoring the course of an outbreak
- Detection of "cholera hotspots"
- Routine surveillance of cholera in endemic areas to characterize its epidemiology
- Detection of high-risk groups
- Monitoring the effectiveness of cholera prevention programs

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[1] As a replacement for Cary-Blair transport medium, some use a filter paper method. The soiled filter paper is put into a cryovial and three drops of saline is added to the vial. The vial is then sent to the laboratory at room temperature (assuming the temperature is < 40°C). This wet filter paper method preserves the bacteria less well than Cary Blair, but can be used if Cary Blair is not available.

[2] For more information: Laboratory Methods for the Diagnosis of Epidemic Dysentery and Cholera. Centers for Disease Control and Prevention, 1999. http://www.cdc.gov/cholera/pdf/Laboratory-Methods-for-the-Diagnosis-of-Epidemic-Dysentery-and-Cholera.pdf

Ideally, microbiological surveillance, as described in this manual, should occur within the framework of an epidemiological surveillance system to enable the results to be extrapolated to a larger population. Additional information on surveillance is found in The StopCholera Toolkit.

A description of the direct and enriched dipstick test kit

The dipstick test method described in this manual makes use of the Crystal VC test kit (16IC101-10, Span Diagnostics, Surat, India), which is the rapid test for cholera that has been used the most in the field. This test, which costs USD $1.90 each when purchased in bulk, uses monoclonal antibodies specific for the lipopolysaccharides (LPS) of *V. cholerae* serogroups O1 and O139 in a vertical flow immunochromatography dipstick. The LPS detection level of the kit is 10 ng/ml for VC O1 and 50 ng/ml for VC O139. A recent study reported that the minimum detectable limit of the kit was 10^6 Colony Forming Units (CFU) of VC O1/ml and 10^7 CFU of VC O139/ml [1]. Our laboratory found that about 10^7 CFU/ml were needed for both serogroups to yield a positive test [2].

Crystal VC has separate lines for serotype O1 and O139. In the past, a kit with only the O1 line was available and there are plans to market this product again. The test with the single line for serotype O1 is preferred since serotype O139 is almost never seen, but false positive results may occur with the O139 line and may be confusing.

Each dipstick has a control line to ensure that the test is able to detect the antigen. The kit can be stored in temperatures ranging between 4° and 30°C and in humid conditions. It is not approved by the U.S. Food and Drug Administration (FDA), but it is marketed in India and has been used in several field studies. According to published reports, the dipstick test has a sensitivity of about 90% and a specificity of about 60–70% when used directly on patients' fecal specimens [3, 4]. The relatively low specificity suggests that an improvement in the test procedure is needed to ensure that a positive test represents a true positive case. Especially in areas without known cases of cholera, declaring a cholera outbreak, when in fact there is no cholera, might result in resources being devoted inappropriately. Even in many cholera-endemic areas, the low specificity yields a positive predictive value of less than 50%.

The reasons for false positive results with the rapid test when used directly with the fecal sample are not known. In some cases, fecal samples may contain materials (other than *V. cholerae*) that cross-react with the antibody and show up on the test as a positive line. In other cases, technicians without sufficient training or experience may not exactly follow the test instructions in the product insert. They may attribute a faint line as being positive or they may evaluate the test after the prescribed 15 minute time limit. In other instances, the dipstick test may be giving a true reading with a specimen that the culture missed because the patient had taken antibiotics. In this latter situation, the "apparent false positive" result is actually a true positive even though it was not confirmed by culture. In the future, PCR will likely be considered the gold standard method against which to compare other tests.

Direct Test: When the test is used directly with diarrheal stool, two drops of liquid stool are placed into a vial with the buffer provided with the kit and mixed together. Four drops of the stool-buffer mix is placed into a test tube which is provided and the dipstick is then placed vertically into the tube. The liquid from the test tube can be observed to move up the dipstick. If *V. cholerae* is not present, a single (control) line will appear within a few minutes. If *V. cholerae* is present, two lines will appear. The lines will be apparent within 15 minutes, and generally within five minutes.

Enriched Test: The enriched (or confirmatory) method is similar to the direct method except that a small amount of the stool specimen is first placed into a broth of alkaline peptone water (APW) and incubated for five to 18 hours. After the incubation at 20-40°C, four drops of the APW are placed into the test tube and the dipstick is placed vertically into the tube. If *V. cholerae* is present, two lines will appear, including the control line and the test line. *V. cholerae* grows well at a wide range of temperatures so that an incubator is not necessary in most tropical countries where cholera occurs. However, the temperature should not go above 40°C. Therefore, in very hot areas, methods may be needed to keep the temperature below 40°C.

When the fecal sample is incubated in the APW, the cross-reacting material (if present) is diluted so much that it no longer causes a false positive line to show up on the dipstick. At the same time, the *V. cholerae* bacteria grow and the LPS antigen is amplified. As a result of these two factors, the test is less likely to yield a false positive and

should provide a more accurate result. When used in this manner, the specificity of the test in Cameroon was >99% [5].

The decision about whether to use the direct or the enriched test depends on logistic and scientific considerations. The direct test provides a very rapid result which may be especially helpful for patients arriving in the afternoon and evening. If the result is negative, the test is generally reliable. However, if the test is positive when tested with the stool specimen directly, this should be followed up with a confirmatory test; either a culture or an enriched test.

The APW enrichment method is a logical procedure based on sound microbiological concepts, but it is still undergoing field-testing to document its sensitivity and specificity in a variety of settings. In addition – as with culture – it still depends on the ability of the bacteria to grow in the APW, which may not occur if the patient has taken antibiotics, and may result in a false negative test.

If there are questions about the accuracy of the test or a need to determine antibiotic sensitivity of the outbreak strain, some specimens can be sent to a central laboratory for culture using transport medium (e.g., Cary-Blair medium). The specimens sent to the central lab can be taken either directly from the fecal specimen or from the APW that yielded the positive dipstick test. Confirming the positive dipstick test is especially important when trying to confirm cases in an area not known to have cholera.

How to test fecal specimens using the enriched dipstick assay

Materials and supplies required

- Fecal specimen or rectal swab
- Specimen ID labels
- Sterile cotton-tipped wooden swabs used to inoculate the fecal specimen into the APW
- Tube rack to hold the APW tubes
- Tubes with 5 ml alkaline peptone water (APW) (the volume is not critical)
- Crystal VC dipstick kit
- Cary-Blair transport medium tube (or a saline filter paper sample)
- Disposable latex gloves
- Biohazard bags or other method for safe disposal
- Chlorine/bleach (if used for decontamination)

The procedure

Either stool specimens or rectal swabs of patients who present to the health facility for treatment of acute watery diarrhea can be tested (the stool specimen must not have been treated with chlorine). A cotton swab with a sufficient amount of feces is inserted into a tube of APW. The wooden stick is then broken, leaving the cotton swab immersed in the APW. The APW tube should then be put in a safe place (an incubator at 35–37˚C if available, but the incubation can be at temperatures from 20-40˚C) for six hours (with a range of five to eight hours).

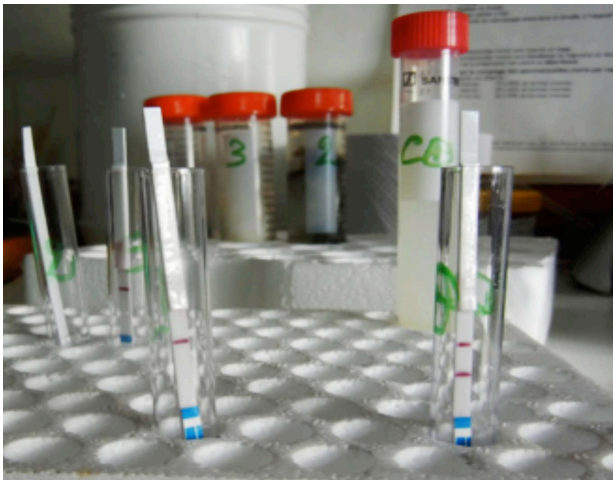
After incubation, place four drops of the APW into the kit test tube and place a Crystal VC dipstick into the test tube. Leave the dipstick in the test tube and see if lines develop on the control line as well as the test line as shown in Figure 1. A positive line will generally be visible within five to ten minutes. Do not record lines that appear after 15 minutes.

To validate the dipstick test, some of the positive samples should be sent to a microbiology laboratory for confirmation by culture following the procedure shown in Figure 2. This chart shows that when a positive result is found, the original fecal sample, as well as a sample from the APW, can be sent to the laboratory for culture

by placing a small amount of the specimen in Cary-Blair transport medium. These specimens should be collected and sent promptly to the lab, but once in transport medium, they will be valid for up to a week or longer.

The number of specimens to send to the microbiology lab depends on the situation. During a confirmed outbreak, only a few specimens need to be sent in order to confirm the diagnosis and to test for antibiotic sensitivity. If an unexpected outbreak of diarrhea is occurring, or if sporadic cases are being detected, a higher proportion of positive cases (10–20%) should be confirmed by the lab to ensure that these specimens are not false positives.

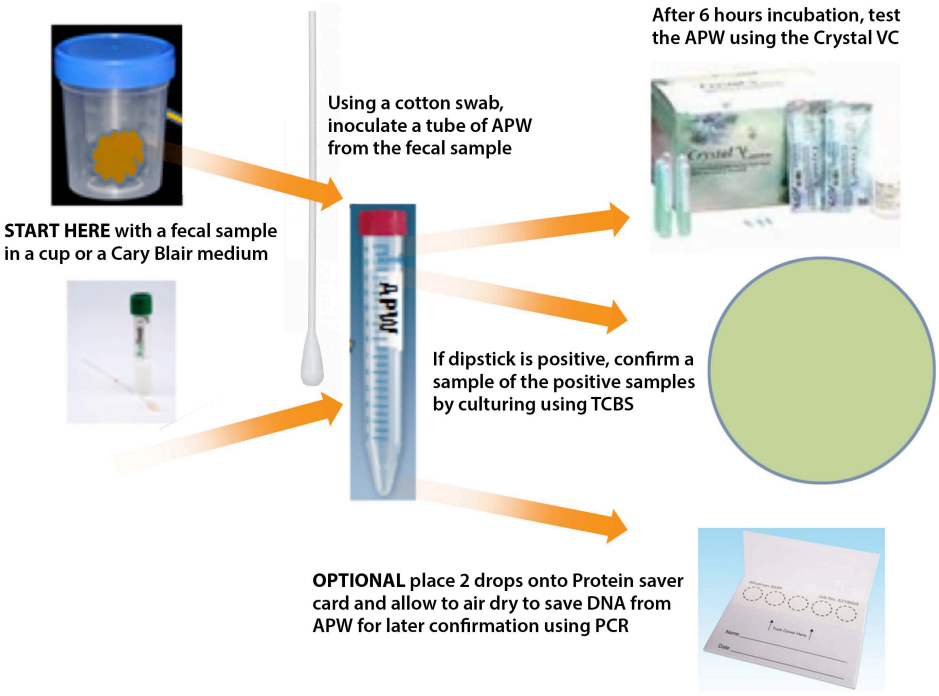
Figure 1. The upper line on each sample is the control line. The lower line is the positive line. If the control line is not seen, the test is not valid.



Negative sample is on the left. The positive sample is on the right.

A positive sample

Figure 2. Procedure for detecting *V. cholerae* O1 from a fecal specimen using dipstick method



Safe Handling and Disposal of Specimens

Please note that the specimens and the APW are biohazards. They need to be discarded safely, according to local procedures for hospital-contaminated waste. Universal precautions in the laboratory need to be observed, including the use of protective clothing and proper decontamination of materials. If the specimens or their derivatives are to be shipped to other laboratories, precautions must be taken for safe transfer of materials using packaging recommended for category B specimens. This includes the triple packaging system with a primary waterproof and leak-proof receptacle containing the specimen. This receptacle is surrounded by absorbent material in case of leakage. The primary package is then contained within a second waterproof and leak-proof receptacle. Several specimens wrapped in primary receptacles can be transported in the same secondary receptacle. Finally the third receptacle is the outer shipping package that can protect the inner contents from damage while being transported.

Conclusion

The modified dipstick technique for *V. cholerae* O1 detection described here has a number of advantages. It is:

1. Low cost,
2. Easy to use, and
3. Reduces the probability of false positives compared with other methods.

Therefore, this low cost and simple method for detecting *V. cholerae* O1 using the modified dipstick technique described in this manual has the potential to greatly improve the feasibility and reliability of cholera surveillance in countries where it is most needed.

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5. The decision about whether to use the direct or the enriched test depends on logistic and scientific considerations. The direct test provides a very rapid result which may be especially helpful for patients arriving in the afternoon and evening. If the result is negative, the test is generally reliable. However, if the test is positive when tested with the stool specimen directly, this should be followed up with a confirmatory test; either a culture or an enriched test.
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