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A Novel Kit for Rapid Detection of *Vibrio cholerae* O1

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We report on the development and testing of a novel, rapid, colorimetric immunodiagnostic kit, Cholera SMART, for direct detection of the presence of *Vibrio cholerae* O1 in clinical specimens. Unlike conventional culture methods requiring several days to complete, the Cholera SMART kit can be used directly in the field by untrained or minimally skilled personnel to detect *V. cholerae* O1 in less than 15 min, without cumbersome laboratory equipment. A total of 120 clinical and environmental bacterial strains, including both O1 and non-O1 serotypes of *V. cholerae* isolated from samples collected from a variety of geographical regions, were tested, and positive reactions were observed only with *V. cholerae* O1. Also, results of a field trial in Bangladesh, employing Cholera SMART, showed 100% specificity and 96% sensitivity compared with conventional culture methods. Another field trial, in Mexico, showed that Cholera SMART was 100% in agreement with a recently described coagglutination test when 108 stool specimens were tested.

Cholera, a life-threatening diarrheal disease, continues to devastate many developing countries, with immense global implications (4). The causative agent of cholera is a gram-negative bacterium, *Vibrio cholerae*, and is generally transmitted to humans via contaminated water and food. By June 1993, at least 830,000 cases of cholera, with more than 7,200 fatalities, had occurred in 20 Latin American countries since the most recent epidemic began in Peru in January 1991 (17). Because of the clinical and epidemiological severity of cholera, it is critical to determine as quickly as possible the presence of *V. cholerae* O1 in clinical specimens, water, and food so that appropriate monitoring and effective preventive measures can be undertaken by public health authorities (4, 12).

Conventional culture methods currently available for detection of *V. cholerae* O1 are time-consuming and expensive and lack the necessary sensitivity, particularly for clinical samples from patients previously treated with antibiotics and/or traditional home remedies or for poorly handled samples. Also, if the number of viable organisms is low, detection will be difficult, especially in some environmental samples where *V. cholerae* O1 can exist in a metabolic state such that it cannot be cultured by conventional methods but remains viable and capable of producing disease (3, 4, 6).

Several polyclonal-antibody-based coagglutination tests for *V. cholerae* O1 have been described previously, but these methods require either cross-absorption with staphylococci and centrifugation (10), a 4-h enrichment (18), or overnight culturing on primary isolation plates (19). Recently, the PCR has been reported to be a reliable and sensitive method for detection of toxigenic *V. cholerae* O1 strains (7, 14, 21). However, PCR requires trained personnel and sophisticated equipment, which are generally not available in all areas of the world, particularly where cholera is a serious and recurrent problem.

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Colwell et al. (4) recently reported the development and evaluation of a rapid, simple coagglutination test, CholeraScreen (New Horizons Diagnostics [NHD] Corporation, Columbia, Md.). The CholeraScreen test employs a monoclonal antibody, COLTA (University of Maryland Biotechnology Institute, College Park, Md.), specific to the A factor of *V. cholerae* O1 lipopolysaccharide. This monoclonal antibody has been found to be highly specific for *V. cholerae* O1 when tested by the indirect fluorescent-antibody method (2, 6).

Reported here are the development and testing of a colloidal-gold-based, colorimetric immunoassay employing NHD Sensitive Membrane Antigen Rapid Test (SMART) technology for direct detection of the presence of *V. cholerae* O1 in clinical specimens. The COLTA monoclonal antibody (6) and a high-titer polyclonal anti-*V. cholerae* O1 antibody (1) have been incorporated into the SMART kit to produce a novel, rapid, simple method for detection of *V. cholerae* O1. The principle of the test is as follows. A specimen suspected of containing *V. cholerae* O1 is reacted to a colloidal-gold-labeled monoclonal antibody, COLTA (University of Maryland Biotechnology Institute). If *V. cholerae* O1 antigen is present in a specimen, it complexes to the anti-*V. cholerae* O1 monoclonal antibody and the complex diffuses and is subsequently captured and concentrated by a polyclonal-antibody-coated solid-phase matrix and appears to the naked eye as a pink-to-red test spot developing from the deposition of colloidal gold. In the absence of *V. cholerae* O1, a complex does not form and, hence, no pink-to-red color appears in the test spot (9).

Development of the Cholera SMART kit. In the Cholera SMART format employed here, the antigens are bound within a monoclonal-polyclonal-antibody sandwich. In the development phase of the diagnostic Cholera SMART kit, other monoclonal antibodies, as well as other combinations of antibodies, were tested in the sandwich assay, including monoclonal-monoclonal- and polyclonal-polyclonal-antibody pairings. The combination presented in this study proved the most effective on the basis of sensitivity and specificity, as well as color intensity (9).

The Cholera SMART kit consists of a reaction vial and a SMART device. The reaction vial contains a lyophilized colloidal-gold-labeled monoclonal antibody, COLTA (Maryland Biotechnology Institute). The SMART device comprises two compartments. The upper compartment contains a membrane bounded by an area large enough to hold the swab used to transfer the test specimen from the reaction vial. The lower compartment contains two immunoglobulin-coated spots (a test spot coated with anti-*V. cholerae* O1 polyclonal antibody (1) and a negative control spot coated with a normal rabbit immunoglobulin). The polyclonal anti-*V. cholerae* O1 antisera were produced in rabbits immunized with 10^9 CFU of a mixture of whole, heat-killed cells of the Classical Ogawa (ATCC 14035) and El Tor Inaba (LA 5875) strains of *V. cholerae* by the method of Sakazaki et al. (20). The specificity and antibody activity in each preparation of anti-*V. cholerae* O1 serum were tested by both slide agglutination (1) and coagglutination (4). The kit contains reconstitution buffer (0.01 M phosphate-buffered saline containing 3% Tween 20, pH 7.3), extraction buffer (0.05 M Tris, 0.05 M EDTA, 0.1 M NaCl, pH 8.2), and a specimen-filtering device consisting of a squeezable 2-ml plastic tube with a snap-on filter (1-μm pore size) that allows bacterial cells to pass through while retaining any particulates that could interfere with the reaction. In addition, swabs (100% Dacron) for reagent transfer or sampling are also provided in the kit.

Procedure. Specimens were treated in accordance with the instructions of the manufacturer (NHD). In brief, 2 drops of reconstitution buffer are added first into the reaction vial. Then 4 drops of a watery stool specimen are released through the filtering device, provided as part of the kit, into the reaction vial. If the stool specimen is not in liquid form (e.g., at least semisolid), it is treated with extraction buffer in the filtering device before 4 drops are released into the reaction vial. A sterile swab is inserted into the reaction vial to absorb the reconstituted contents. The swab is then placed in the upper compartment of the SMART device, after which the device is closed. The reactants diffuse from the swab through the membrane onto both the test and negative control spots in the lower compartment of the kit.

If *V. cholerae* O1 antigen is present, the antigen-antibody complex formed with colloidal gold is captured by the polyclonal anti-*V. cholerae* O1 antibody in the lower compartment. A red dot appears within 5 to 10 min, whereas the negative control spot remains unchanged, i.e., no color develops. If the antigen is absent or below the level of sensitivity of the test kit, the test spot does not develop any color visible to the naked eye. From start to finish, the test is performed in less than 15 min.

Specificity of Cholera SMART. The specificity of the kit was determined by using 120 pure cultures isolated from samples collected in several geographical regions of the world and from various sources. These bacterial strains were selected to represent the families of *Vibrionaceae*, *Enterobacteriaceae*, and *Aeromonadaceae* (5). In brief, colonies of a pure culture grown on tryptic soy agar (Difco Laboratories, Detroit, Mich.) were suspended in sterile saline (0.15 M NaCl) to McFarland no. 1 standard (3×10^8 CFU/ml) (15). Two drops of reconstitution buffer and 4 drops of culture fluid were added to the reaction vial. The test swab was inserted into the reaction vial and allowed to absorb the contents, after which the swab was placed in the SMART device as described above. Results were obtained within 10 min and recorded.

Sensitivity of Cholera SMART. Tenfold serial dilutions of a known concentration (ca. 6.0×10^8 CFU/ml) of *V. cholerae* O1 were used to determine the sensitivity of the test. The known concentration of *V. cholerae* O1 was determined by adjusting the concentration to a level equivalent to a McFarland no. 2 standard ($A_{650} = 0.31$) and confirming the concentration by both plate count on tryptic soy agar and direct fluorescent-antibody staining, using epifluorescence microscopy, in accordance with the manufacturer's (NHD) instructions.

Field trials. (i) Bangladesh. A field trial was conducted at the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDRB) at Dhaka, Bangladesh.

Forty-four diarrheal stool specimens from patients who had some to severe cholera-like symptoms and had been admitted to the clinical research center of the ICDDRB were tested by conventional culture methods as follows. Stool specimens were inoculated onto thiosulfate-citrate-bile salts-sucrose agar (Eiken Chemical Co. Ltd., Tokyo, Japan) and taurocholate-tellurate-gelatin agar (16). Suspected colonies resembling *V. cholerae*, i.e., yellow colonies on thiosulfate-citrate-bile salts-sucrose agar and gelatinase-positive (halo-positive) colonies from taurocholate-tellurate-gelatin agar, were tested by slide agglutination with *V. cholerae* O1 polyvalent antiserum prepared at the ICDDRB. The stool specimens were also tested directly with Cholera SMART and CholeraScreen (the latter is a coagglutination test described elsewhere [4]) by following the manufacturer's (NHD) instructions.

(ii) Mexico. A second field trial was conducted at the Social Security Hospitals in Mexico City and Puebla, Mexico. Of the 108 stool specimens tested, 98 were swabs in Cary-Blair transport medium and 10 were freshly collected specimens. The swabs were treated with extraction buffer in the filtering device and tested directly with Cholera SMART and CholeraScreen.

When tested for specificity, Cholera SMART was positive only with strains of *V. cholerae* O1 ($n = 30$) and did not cross-react with any other bacterial species ($n = 90$), demonstrating 100% specificity (Table 1). In Table 2, the biotype, serotype, and source of each of the 30 strains of *V. cholerae* O1 tested with Cholera SMART kit are presented. From the results obtained with dilutions of pure cultures of *V. cholerae*, it was concluded that a minimum of ca. 6.0×10^6 CFU/ml is required to give an unequivocally positive reaction. The sensitivity of Cholera SMART was at least 1 to 2 orders of magnitude greater when extracted antigens were used.

Forty-four stool specimens were tested in the field trial at the ICDDRB. Twenty-two (95.6%) of the 23 culture-positive (all were El Tor Ogawa) stool specimens were *V. cholerae* O1 positive by Cholera SMART, while the remaining 21 specimens were negative by both culture and Cholera SMART, yielding 100% specificity. The results showed that Cholera SMART detected *V. cholerae* O1 in all of the stool specimens that were culture positive, and none of the negative specimens tested positive ($P < 10^{-8}$; chi-square test). Only one culture-positive, Cholera SMART-negative stool specimen was Cholera SMART positive when subsequently tested with the pure culture isolated from the same stool specimen, suggesting that the number of organisms in the original stool specimen was below the level of detection, i.e., $< 6 \times 10^6$ CFU/ml. Results obtained by Cholera SMART, when compared with those obtained by the culture method, were in agreement (100%) with results of the CholeraScreen (NHD) coagglutination test (4). Of the 108 stool specimens tested in Mexico, 45 were positive by

TABLE 1. Specificity of the Cholera SMART kit for *V. cholerae* O1

Species	No. of strains tested	% Positive
V. cholerae O1[a]	30	100
V. cholerae non-O1	20	0
Aeromonas hydrophila	5	0
A. veronii bv. sobria	2	0
A. caviae	2	0
Shigella dysenteriae	2	0
S. boydii	1	0
S. flexneri	1	0
S. sonnei	1	0
Escherichia coli	3	0
Salmonella typhimurium	2	0
S. thompson	1	0
S. hadar	1	0
S. berta	1	0
S. johannesburg	1	0
Klebsiella pneumoniae	2	0
Serratia marcescens	2	0
Citrobacter freundii	2	0
Proteus vulgaris	2	0
Enterobacter aerogenes	2	0
V. vulnificus	4	0
V. parahaemolyticus	4	0
V. mimicus	10	0
V. alginolyticus	2	0
V. campbelli	2	0
V. fluvialis	3	0
V. damsela	2	0
V. natriegens	2	0
V. pelagius	2	0
V. proteolyticus	2	0
Pseudomonas aeruginosa	2	0
Plesiomonas shigelloides	2	0

[a] The source, biotype, and serotype of each strain of *V. cholerae* O1 are in Table 2.

TABLE 2. Biotypes, serotypes, and sources of 30 *V. cholerae* O1 strains tested with the Cholera SMART kit

Strain	Serotype	Biotype	Source
14033	Inaba	El Tor	ATCC[a]
14035	Ogawa	Classical	ATCC
569B	Inaba	Classical	Stool, India
997313	Ogawa	El Tor	Stool, Bangladesh
997322	Ogawa	El Tor	Stool, Bangladesh
997375	Ogawa	El Tor	Stool, Bangladesh
997380	Ogawa	El Tor	Stool, Bangladesh
DP 1	Inaba	El Tor	Water, United States
DP 3	Inaba	El Tor	Water, United States
DP 5	Inaba	El Tor	Water, Russia
DP 6	Inaba	El Tor	Water, Russia
DP 7	Inaba	El Tor	Water, Ukraine
DP 8	Inaba	El Tor	Water, Ukraine
LL 57	Inaba	El Tor	Stool, Guatemala
LL 58	Inaba	El Tor	Stool, Guatemala
LL 75	Inaba	El Tor	Stool, Guatemala
LL 76	Inaba	El Tor	Stool, Guatemala
LL 81	Inaba	El Tor	Stool, Guatemala
LL 82	Inaba	El Tor	Stool, Guatemala
LL 83	Inaba	El Tor	Stool, Guatemala
LL 87	Inaba	El Tor	Stool, Guatemala
LL 88	Inaba	El Tor	Stool, Guatemala
LL 96	Inaba	El Tor	Stool, Guatemala
LL 97	Inaba	El Tor	Stool, Guatemala
LL 99	Inaba	El Tor	Stool, Guatemala
RG 002	Inaba	El Tor	Stool, Peru
RG 004	Inaba	El Tor	Stool, Peru
RG 007	Inaba	El Tor	Stool, Peru
JH 332	Inaba	El Tor	Stool, Mexico
JH 336	Inaba	El Tor	Stool, Mexico

[a] ATCC, American Type Culture Collection.

Cholera SMART and also positive by CholeraScreen (NHD) while the remaining 63 samples were negative by both tests. The conventional culture method was not used for these samples.

The Cholera SMART method offers simplicity and ease of use, minimal hands-on time, rapid turnaround time, unrefrigerated storage, no need for any additional equipment, and satisfactory deployment under field conditions. The test kit can be stored for up to a year at room temperature without loss of activity. Once the lyophilized colloidal-gold-labeled monoclonal antibody is reconstituted in the reaction vial, it should be used immediately to prevent any loss of activity.

SMART technology (NHD) has already been employed successfully to detect other pathogens, e.g., *Neisseria gonorrhoeae* (13) and *Streptococcus pyogenes* (11) (group A streptococci). The Cholera SMART test also has been introduced in the microbiology laboratory at the University of Maryland as a routine identification procedure and has proven to be effective when compared with labor-intensive biochemical tests.

The recent epidemics of cholera in Central and South America emphasize the need for a rapid, simple, and reliable means of detecting *V. cholerae* O1 for diagnostic purposes, i.e., food and water quality assurance and environmental monitoring, as well as testing of clinical specimens (4, 14). Cholera SMART, a significant development in methodology for rapid detection of *V. cholerae* O1, appears to satisfy this need (8, 9, 22). Unlike conventional culture methods, which take several days to complete, Cholera SMART can detect the presence of *V. cholerae* O1 directly and reliably within minutes in clinical specimens, on site and without cumbersome laboratory equipment and/or trained personnel.

We conclude that Cholera SMART offers a rapid, simple, and reliable alternative to methods currently employed in microbiology laboratories and can be used as a diagnostic tool on site in the field in all areas of the world where cholera is epidemic.

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ADDENDUM IN PROOF

Recently a monoclonal antibody against *Vibrio cholerae* O:139 has been prepared, and a Cholera SMART method for this strain is now possible.

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