DEVELOPMENT OF A 5-MINUTE RAPID TEST FOR DETECTING *VIBRIO CHOLERAE* O139

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Abstract. *Vibrio cholerae* O139 is an etiology of cholera in Thailand. We determined to prepare a rapid test to detect *V. cholerae* O139 using an immunochromatographic method to be used for surveillance and use in community laboratories. We conjugated murine monoclonal antibodies specific to the lipopolysaccharide of *V. cholerae* O139 with colloidal gold particles. The sensitivity of the test was determined using 10-fold dilutions of *V. cholerae* O139. The lowest number of bacterial cells detected by the test was 10^6 cfu/ml. The specificity was determined using 51 isolates of pure cultures, including *V. cholerae* serogroups O1, O139, and non-O1/non-O139 and other enteric bacteria; 27 rectal swab specimens and 100 specimens of enriched alkaline peptone water, 50 of which contained *V. cholerae* O1 and the other did not contain *V. cholerae* O1. The rapid test showed specificity to *V. cholerae* O139 and no cross reaction with *V. cholerae* O1, *V. cholerae* non-O1/non-O139, and other enteric bacteria. The test is rapid, simple and easy to use.

Keywords: *Vibrio cholerae* O139, cholera, rapid test, immunochromatographic test, lateral flow

INTRODUCTION

Vibrio cholerae O139 emerged as an etiologic agent of cholera in India in late 1992 (Cholera Working Group, 1993), and spread to several countries in Southeast Asia, including Thailand (Chongsanguan *et al*, 1993). One hundred forty-three cases of *V. cholerae* O139 infection were confirmed by the National Institute of Health of Thailand in 1993 and 3,024 were confirmed in 1994 (Supawat *et al*, 1997). Cholera outbreaks usually occur in crowded areas where individual hygiene and sanitation are poor or below standard levels (Murray *et al*, 2002), particularly in developing countries. Imported cases have also been reported in developed countries (Sakaue *et al*, 1995; Taniguchi *et al*, 2008). *V. cholerae* O139 infection is rarely reported in Thailand (Bureau of Epidemiology, 2011). The disease has the potential risk to re-emerge.

Cholera infection can be diagnosed by conventional culture, by isolation on specific culture media, by identification with biochemical reactions and by specific antisera. Alternative methods, such as

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polymerase chain reaction (PCR) (Albert *et al*, 1997), loop-mediated isothermal amplification (Okada *et al*, 2010), co-agglutination (Hanumanthappa and Rajagopal, 2001; Islam *et al*, 2004), and immunochromatographic testing (IC) (Harris *et al*, 2009) have been developed and some have been field evaluated (Rohani *et al*, 1998). Of the above-mentioned methods, co-agglutination and IC are performed without the need of any special equipment. The results for co-agglutination and IC are obtained in 3-5 minutes and 2-20 minutes, respectively. These are methods of choice for community laboratories during outbreaks and surveillance purposes.

In this study, we produced antibodies and developed an IC test to detect *V. cholerae* O139. This test uses antigen-antibody interaction on a test line on a nitrocellulose membrane. Results are seen by the accumulation of conjugated colloidal gold-antibody at that site.

MATERIALS AND METHODS

Samples

Fifty-one isolates of enteric bacteria (22 reference isolates and 29 laboratory isolates) (Table 2), 27 rectal swab specimens (Table 3) and 100 enriched alkaline peptone water (APW) samples were used in this study. The enriched APW samples were composed of 50 samples containing *V. cholerae* O1, Ogawa and another 50 samples without *V. cholerae* O1. These enriched APW samples were kindly supplied by Mae Sot Hospital.

Polyclonal antibodies to *V. cholerae* O139

Polyclonal antibodies (PAb) to *V. cholerae* O139 were prepared following guidelines for the production of PAb (Animal Care and Use Committee, 2006) against formalin-treated *V. cholerae* O139 (DMST 16265). Fifty milliliters of rabbit sera containing high antibody titers against *V. cholerae* O139 were collected. The PAb was then partially purified with 40% saturated ammonium sulfate precipitation and the protein content was measured by Lowry's method (Lowry *et al*, 1951). The PAb was used as captive antibody and sprayed on the test line of a nitrocellulose test membrane.

Anti-mouse immunoglobulin

Anti-mouse immunoglobulin (Ig) was prepared following the procedure to prepare PAb against *V. cholerae* O139; a rabbit was immunized with mouse Ig (Sigma Aldrich, St Louis MO). After obtaining a suitable antibody titer, the rabbit's serum was obtained to use as a source of anti-mouse Ig. The antibodies were partially purified and the protein content was measured following the same procedure used for preparing PAb against *V. cholerae* O139. The anti-mouse Ig was used as a built-in control and sprayed on the control line of a nitrocellulose test membrane.

Monoclonal antibodies against *V. cholerae* O139

Hybridoma clone NHV4 (IgG3, k) secreting monoclonal antibody (MAb) specific to *V. cholerae* O139 lipopolysaccharide (Thattiyaphong *et al*, 1999) was used in this study. The antibody was purified by protein G affinity chromatography. The protein content was determined using the same procedure as that used for PAb.

Preparation of colloidal gold particles

Colloidal gold was prepared by reduction of gold chloride with sodium citrate (Verheijen *et al*, 1998) as follows: 100 ml of 1% gold (III) chloride trihydrate (Sigma Aldrich, St Louis, MO) in $DdiH_2O$ was heated in 1 liter of boiling water for 2 minutes and 21.5 ml of 1% sodium citrate

was added to the solution. After 3 minutes, the maximum wavelength (λ_{max}) of the solution was scanned using DdiH$_2$O as a reagent blank. The λ_{max} was 519-520 nm.

Preparation of conjugated colloidal gold–anti-VCO139 MAb

Ten micrograms of MAb against *V. cholerae* O139 was conjugated with 1 ml of colloidal gold, and gently mixed for 10 minutes. Bovine serum albumin (BSA) was added to make a 1% concentration and then the solution was centrifuged at 15,500*g*. The pellet was dissolved with passive gold diluent (2% of 0.05M $NaHPO_4$, 1% BSA) to obtain an optical density (OD) of 30. The conjugate solution was stabilized with 2% sucrose and 8% trehalose and sprayed on a conjugate releasing pad (CFCP203000, Millipore, Billerica, MA) at a speed of 10 µl/cm using Biodot BJQ 3000 model XYZ 3200.

Control line and test line

Two mg/ml of PAb against *V. cholereae* O139 and 4 mg/ml of anti-mouse Ig were sprayed on a nitrocellulose membrane (AE 100, Whatman) at the test and control lines at a speed of 1.0 µl/cm and 0.8 µl/cm, respectively, using the Biodot BJQ 3000 model XYZ 3200.

Preparation of the test

The test strip was constructed and put in a cassette; its appearance is shown in Fig 1. One hundred microliters of each specimen was placed in a rapid test and left at room temperature for 5 minutes; a red colored band at the control and test lines in a positive sample and at the control line only in a negative sample. Each test dilution was performed in triplicate.

The antigenic sensitivity or limit of detection (LOD) for the rapid test was determined using *V. cholerae* O139 ATCC 51394 with a known bacterial concentration. A ten-fold dilution of *V. cholerae* O139 was made by diluting the organism in solution ranging in concentration from $1x10^4$ to $1x10^8$ cfu ml^{-1} in 4 different solutions: 0.1 M phosphate buffer saline (PBS) pH 7.4; APW, PBS containing 3% BSA, 1% gelatin, 0.05% Tween 20; and APW containing rectal swabs.

The sensitivity and cross reactivity of the test were determined using 51 reference strains (Table 2), and 27 rectal swabs.

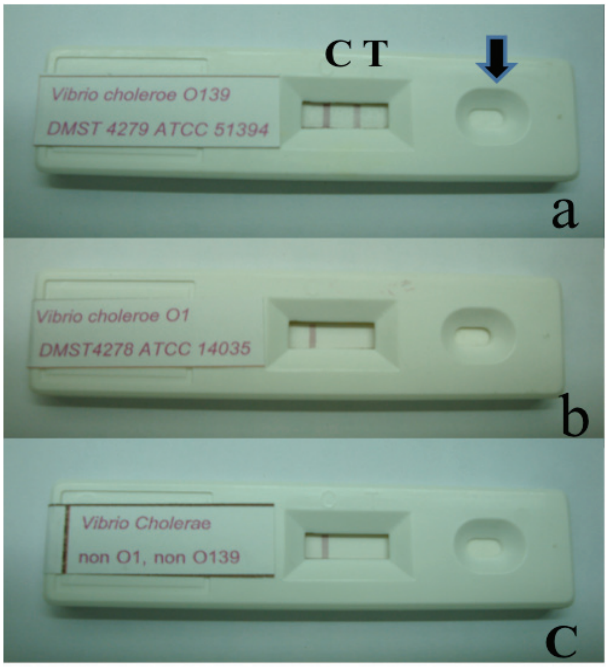

Fig 1– IC test for detecting *V. cholerae* O139; 100 µl of sample is dropped into the sample well indicated by the arrow, (Fig 1a) followed by allowing the sample to set for 5 minutes before reading the results. Positive results are indicated by observing 2 red bands: a test line (T) and a control line (C); negative results are indicated by observing only one band. Fig 1b shows a negative result for *V. cholerae* O1 ATCC 14035 and Fig 1c shows a negative result for *V. cholerae* nonO1/ nonO139.

Table 1
Test results for *V. cholerae* O139 at various concentrations in different solutions.

Solution	VCO139 (cfu/ml)				
	10^4	10^5	10^6	10^7	10^8
0.1 M PBS pH 7.4	-	+	+	+	+
APW	-	+	+	+	+
0.1 M PBS pH 7.4 + BSA + gelatin + Tween 20	-	+	+	+	+
APW +RS1	-	-	+	+	+
APW +RS2	-	-	+	+	+

VCO139, *Vibrio cholerae* O139; PBS, phosphate buffer saline; APW, alkaline peptone water; BSA, bovine serum albumin; RS, rectal swab; +, positive result; - , negative result.

Each reference strain was sub-cultured on Trypticase Soy Agar (TSA) overnight and one colony each was selected to culture in 5 ml of APW for 2, 4, and 6 hours. At the end of each incubation time, the cultures were tested with a rapid test. The 27 rectal swabs were enriched in APW and then the organisms were isolated and identified by conventional methods. The enriched APW samples were tested with the rapid test. In addition, 100 enriched APW samples either with or without *V. cholerae* O1 were also tested with the rapid test.

RESULTS

The rapid test detected a reference strain of *V. cholerae* O139 diluted in 3 diluents: 1) 0.1 M PBS pH 7.4; 2) APW; and 3) 0.1 M PBS pH 7.4 containing BSA, gelatin and Tween 20 at concentrations of 10^5, 10^6, 10^7 and 10^8 cfu/ml. Rectal swab specimens were determined in APW at concentrations of 10^6, 10^7 and 10^8 cfu/ml (Table 1). The 51 isolate samples placed in APW for 2, 4 and 6 hours were examined with the rapid test; only *V. cholerae* O139 gave positive results at all incubation times, the other organisms gave negative results on the rapid test (Table 2).

The bacteria obtained from the 27 rectal swabs were isolated and identified by conventional culture technique (Table 3). No *V. cholerae* O139 organisms were isolated. These specimens were all examined with the rapid test and all gave negative results.

The rapid test was used to detect *V. cholerae* O1, Ogawa in the 100 APW samples, 50 containing *V. cholerae* O1, Ogawa and the rest did not. All samples gave negative results. (This means the rapid test did not detect *V. cholerae* O1, Ogawa, our rapid test detected only *V. cholerae* O139).

DISCUSSION

The lowest concentration of *V. cholerae* O139 detected with the rapid test was 10^5 cfu/ml. *V. cholerae* O139 was detected in specimens containing fecal materials and other bacteria at a concentration of 10^6 cfu/ml. Fecal material and other bacteria interfered with the sensitivity of the test.

The stool of patients infected with *V. cholerae* contain concentrations of 10^7 to 10^8 cfu/g (Deshpande, 2002); therefore, this rapid test is suitable for detecting clinical specimens without enrichment.

Table 2
Cross-reactivity between *V. cholerae* O139 and various strains of enteric bacteria using the rapid test.

No.	Organism	No. of isolates	Results		
			2 h	4 h	6 h
1.	*Vibrio cholerae* O139 ATCC 51394	1	+	+	+
2.	*Vibrio cholerae* O139 (lab)	3	+	+	+
3.	*Vibrio cholerae* O1 ATCC 14035	1	-	-	-
4.	*Vibrio cholerae* O1(lab)	3	-	-	-
5.	*Vibrio cholerae* non-O1/non-O139 (lab)	4	-	-	-
6.	*Vibrio furnissii* (lab)	1	-	-	-
7.	*Vibrio fluvialis* (lab)	2	-	-	-
8.	*Vibrio parahaemolyticus* (lab)	3	-	-	-
9.	*Vibrio alginolyticus* (lab)	1	-	-	-
10.	*Salmonella* Havana DMST 7116	1	nd	-	-
11.	*Salmonella* Urbana DMST 10881	1	nd	-	-
12.	*Salmonella* Typhimurium ATCC 13350	1	nd	-	-
13.	*Salmonella* Derby DMST 16881	1	nd	-	-
14.	*Salmonella* Wandsworth DMST 19204	1	nd	-	-
15.	*Salmonella* group C (lab)	2	-	-	-
16.	*Salmonella* group E (lab)	2	-	-	-
17.	*Salmonella* group B (lab)	1	-	-	-
18.	*Escherichia coli* ATCC 35218	1	nd	-	-
19.	*Escherichia coli* DMST 25922	1	nd	-	-
20.	*E. coli* O157: H7 DMST 12743	1	-	-	-
21.	STEC *E. coli* DMST 30539	1	-	-	-
22.	ETEC DMST 30543	1	-	-	-
23.	EPEC DMST 30548	1	-	-	-
24	EIEC DMST 20971	1	-	-	-
25.	*S. dysenteriae* DMST 7123	1	-	-	-
26.	*S. flexneri* DMST 4423	1	-	-	-
27.	*S. sonnei* DMST 17563	1	-	-	-
28.	*E. faecalis* ATCC 29212	1	-	-	-
29.	*C. freundii* DMST 16368	1	-	-	-
30.	*E. tarda* (lab)	4	-	-	-
31.	*S. aureus* ATCC 25923	1	-	-	-
32.	*S. epidermidis* ATCC 12228	1	-	-	-
33.	*Staphylococcus aureus* (lab)	4	-	-	-
	Total	51			

nd, not determined; lab, laboratory strain.

However, the concentration of organisms excreted in the stool of asymptomatic patients is lower (Deshpande, 2002), so enrichment of the stool specimen is needed prior to testing to obtain accurate results.

The sensitivity of the Cholera Spot test is 10^6 cfu/ml (Rohani *et al*, 1998), the

Table 3
Cross-reactivity between *V. cholerae* and other enteric bacteria from rectal swabs enriched in APW using the rapid test.

No.	Bacteria determined by culture method	Rapid test
1	*E. coli*, *Enterobacter* spp, *P. shigelloides*	negative
2	*E. coli, S. aureus*, *Citrobacter* spp, *Enterobacter* spp	negative
3	*E. coli, Citrobacter* spp, *Providencia* spp	negative
4	*Enterobacter* spp	negative
5	*Proteus* spp	negative
6	*Citrobacter* spp, *Enterobacter* spp, *Proteus* spp	negative
7	*Citrobacter* spp	negative
8	*E. coli*	negative
9	*Citrobacter* spp, NFB	negative
10	*E. coli, Providencia* spp	negative
11	*E. coli, B. cereus*	negative
12	*B. cereus*	negative
13	*E. coli, Proteus* spp	negative
14	*E. coli*	negative
15	*A. caviae, Citrobacter* spp	negative
16	*B. cereus*	negative
17	*Citrobacter* spp, *B. cereus*	negative
18	*S. aureus*	negative
19	*E. coli, B. cereus*	negative
20	*E. coli*, *Klebsiella* spp	negative
21	*E. coli*, *S. aureus*	negative
22	*E. coli, Aeromonas sobria, P. shigelloides*	negative
23	*E. coli, A. sobria, Citrobacter* spp, *Enterobacter* spp	negative
24	*E. coli, V. parahaemolyticus, Proteus* spp	negative
25	*E. coli, A. hydrophila, V. parahaemolyticus, Proteus* spp	negative
26	*Enterobacter* spp, *Citrobacter* spp, *V. parahaemolyticus, E. coli*	negative
27	*V. cholerae* non-O1/non-O139	negative

NFB, non-fermentative gram-negative bacteria.

same as our test, but the Cholera Spot test takes 20 minutes to obtain results, our rapid test takes only 5 minutes. The crystal *V. cholerae* rapid dipstick test (Harris *et al*, 2009) also takes 20 minutes to obtain results. The shorter time with our test may be due to the stronger binding between antigen and antibodies.

Our rapid test detected *V. cholerae* O139 at 2, 4 and 6 hours and had no cross reactions with other enteric bacteria (Table 2). Our rapid test was specific for *V. cholerae* O139. There were no false positive specimens among the rectal swabs in our study. The 50 APW samples with and without *V. cholerae* O1 all gave negative results with our rapid test. We did not use specimens containing *V. cholerae* O139 to detect the diagnostic sensitivity of our test.

Our test is rapid and simple to use. Clinical specimens can be directly used without any additional processing if the concentration of *V. cholerae* O139 cells is

$>10^6$ cfu/ml. If the concentration is lower than this, the specimen should be enriched in APW for at least 2-4 hours before testing. Our strip needs to be further tested with actual clinical samples.

Performing our strip test does not require experienced personnel or specific equipment, so it can be used in community hospitals where no microbiology laboratory is available. Using the strip involves minimal labor and expenses. The material cost of each strip is less than THB 25 (USD 0.80), compared to the culture method which cost THB 200-300 (USD 6.45-9.68). The strip could be most effectively used with a strip to detect *V. cholerae* O1, the most common cause of cholera outbreaks. A *V. cholerae* O1 strip has been developed at our institution with the same material cost as the *V. cholerae* O139 strip. The use of these strips can reduce the number of specimens for which a culture needs to be obtained for confirmation. Stool specimens collected from patients and contacts at outbreak sites can be processed at the collection area.

This strip may be used to detect *V. cholerae* O139 in export food samples. Further studies are needed to determine the practicability of using this strip in the clinical setting. This strip model may be useful when developing rapid tests for other enteric pathogens.

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