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Research Letter

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Nanoparticle-based lateral flow biosensor combined with multiple cross displacement amplification for rapid, visual and sensitive detection of *Vibrio cholerae*

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One sentence summary: MCDA-LFB for detection of *Vibrio cholerae*.
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ABSTRACT

Vibrio cholerae is an important human pathogen that is responsible for cholera, a severe acute watery diarrhea. In the current study, a multiple cross displacement amplification (MCDA) coupled with amplicon detection by chromatographic lateral flow biosensor (LFB) method (MCDA-LFB) was successfully established and evaluated for the identification of *V. cholerae*. A set of 10 primers was designed specifically to recognize 10 different regions of the *V. cholerae*-specific gene *ompW*. The optimized time and temperature conditions for the MCDA were 30 min and 63°C, respectively. The MCDA-LFB assay correctly identified 31 strains of *V. cholerae* but did not detect 13 non-*cholerae Vibrio* strains and 30 non-*Vibrio* strains. The sensitivity of MCDA-LFB for target pathogen detection in pure culture was 10 fg per reaction. In the case of spiked shrimp samples without enrichment, the limit of detection was 4.1 CFUs per reaction or equivalent to 4.1×10^2 CFU g^{-1}. The whole process, including shrimp homogenates processing (30 min), MCDA reaction (30 min) and results reporting (2 min), could be finished within 65 min. These results show that this assay is suitable for the rapid, sensitive and specific detection of *V. cholerae* in food, environmental and clinical samples.

Keywords: *Vibro cholerae*; multiple cross displacement amplification; lateral flow biosensor; MCDA-LFB; limit of detection

INTRODUCTION

Vibrio cholerae is the etiological agent of the human disease cholera that is still a serious health problem in many countries, including Haiti, various regions of Africa and much of Asia (Sack *et al.* 2003; Kirigia *et al.* 2009; Fraser 2010; Patra *et al.* 2011). *Vibrio cholerae* is considered as a food- and water-borne human pathogen, and can be divided into two groups: cholera-causing strains of serogroups O1 and O139 and non-O1 non-O139 cholera vibrios (NCVs) (Chatterjee *et al.* 2009). *Vibrio cholerae* belonging to O1 and O139 serogroups has drawn attention because they have the ability to initiate epidemic and pandemic cholera (Sack *et al.* 2004). NCVs are generally regarded as part of the normal

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bacterial flora of estuarine and coastal environments, and have long been recognized to be non-pathogenic organism (Chatterjee *et al.* 2009). In the last few decades, NCVs have been reported to cause sporadic cases or occasional outbreaks of diarrhea in humans (Chatterjee *et al.* 2009; Marin *et al.* 2013; Crowe *et al.* 2016). Therefore, it is imperative to establish a rapid responsive mechanism and strategy for the rapid, sensitive and specific detection of all *V. cholerae*, which may institute adequate therapy, mitigate the scale of the outbreak and facilitate better epidemic prevention.

Currently, the gold standard to detect and identify *V. cholerae* is still the bacterial culture and biochemical methods, which are time consuming, laborious and lack of sensitivity (Dick *et al.* 2012). Although biochemical identification methods are commercially available, these systems may not always be reliable (O'Hara *et al.* 2003). Moreover, several *Vibrio* species exhibit similar biochemical characteristics that hamper the differentiation of species by biochemical tests (Thompson, Austin and Swings 2006; Wang *et al.* 2016b). Many modern diagnostic techniques have been developed, such as ELISA, PCR-based methods, indirect immunofluorescent assay, etc. (Dick *et al.* 2012; Chen *et al.* 2014). Although these approaches are faster than the culture-biotechnical techniques and display much higher sensitivity, they require sophisticated apparatus to analyze the samples and specialized personnel to interpret the results. In particular, many cholera prevalence regions are poor and usually not well equipped with expensive equipment and occupied with highly skilled staffs, seriously limiting the usefulness of these assays in resource-challenged regions.

Recently, a novel nucleic acid amplification technique, termed multiple cross displacement amplification (MCDA), has been successfully developed, and demonstrated high specificity, rapidity and simplicity, yielding amplicons from as few as three bacterial cells (Wang *et al.* 2015). MCDA is based on the principle of autocycling strand displacement synthesis conducted by the *Bst* DNA polymerase, and the reaction is performed under isothermal conditions (58°C–67°C) for 30 to 60 min. In terms of analysis of MCDA products, lateral flow biosensors (LFBs) have been devised to visually detect double-labeled MCDA amplicons in 1 to 2 min (Wang *et al.* 2016b; Wang *et al.* 2017a,b). MCDA coupled with LFB (MCDA-LFB) has been successfully developed to detect specific DNAs from bacterial pathogens, including *Shigella*, *Salmonella*, *Listeria monocytogenes* and *V. parahaemolyticus* (Wang *et al.* 2016a,b, 2017a,b).

In this report, the first MCDA-LFB assay for the detection of all *V. cholerae* was established based on the target sequence of *ompW* gene. The sensitivity and specificity in pure culture and spiked shrimp samples were examined by comparison with that of PCR assay.

MATERIALS AND METHODS

Reagents and instruments

QIAamp DNA Mini Kit (QIAamp DNA minikits; Qiagen, Hilden, Germany) was purchased from Qiagen Co., Ltd (Beijing, China). The Loopamp kits were purchased from Eiken Chemical Co., Ltd (Beijing, China). The visual detection reagent (hydroxynaphthol blue, HNB) was purchased from BeiJing-HaiTaiZhengYuan Technology Co., Ltd (Beijing, China). Nitrocellulose membrane (NC), sample and conjugate pads, absorbent pads and membrane backing materials were purchased from Jie Yi Biotechnology Co., Ltd (Shanghai, China). Biotinylated bovine serum albumin (biotin-BSA) and rabbit anti-fluorescein antibody (anti-FITC Ab) were purchased from the Abcam Co., Ltd (Shanghai, China). Streptavidin-immobilized 30-nm gold nanoparticles (SA-G) were purchased from BeiJing-HaiTaiZhengYuan Technology Co., Ltd.

Table 1. Bacterial strains used in this study.

Bacteria species	Isolates (source)[a]	No. of strains
Vibrio cholera (non-O1/non-O139)	ICDC-NPVc001	1
Vibrio cholera (non-O1/non-O139)	Isolates	10
Vibrio cholera (O1)	Isolates	10
Vibrio cholera (O139)	Isolates	10
Vibrio parahaemolyticus	ICDC-VP001	1
	Isolates	4
Vibrio vnlnificus	ATCC27562	1
	Isolates	4
Vibrio mimicus	Isolate	1
Vibrio fluvialis	Isolate	1
Vibrio alginolyticus	Isolate	1
Listeria monocytogenes	ATCC19114	1
Listeria ivanovii	ATCCBAA-678	1
Listeria grayi	ATCC25402	1
Listeria innocua	Isolate	1
Listeria welshimeri	Isolate	1
Listeria seeligeri	Isolate	1
Enterohemorrhagic *E. coli*	EDL933	1
Enteropathogenic *E. coli*	Isolates	2
Enterotoxigenic *E. coli*	Isolate	1
Enteroaggregative *E. coli*	Isolate	1
Enteroinvasive *E. coli*	Isolate	1
Plesiomonas shigelloides	Isolate	1
Aeromonas hydrophila	Isolate	1
Shigella dysenteriae	Isolate	1
Shigella boydii	Isolate	1
Shigella flexneria	Isolate	1
Shigella sonneri	Isolate	1
Salmonella	Isolate	1
Enterococcus faecalis	ATCC35667	1
Enterococcus faecium	Isolate	1
Staphylococcus aureus	Isolate	1
Staphylococcus epidemidis	Isolate	1
Yersinia enterocolitica	ATCC23715	1
Enterobacter cloacae	Isolate	1
Bntorobater sakazakii	Isolate	1
Bacillus cereus	Isolate	1
Campylobacter jejuni	ATCC33291	1
Pseudomonas aeruginosa	Isolate	1
Klebsiella pneumoniae	Isolate	1

ATCC, American Type Culture Collection; ICDC, National Institute for Communicable Disease Control Disease Control and Prevention, Chinese Center for Disease Control and Prevention.

Bacterial strains, culture conditions and genomic template preparation

A total of 74 bacterial strains, including 31 *Vibrio cholerae* strains, 13 non-*cholerae Vibrio* strains and 30 non-*Vibrio* strains, were used in the current report (Table 1). All bacteria were stored in 10% (w/v) glycerol broth at –70°C. The *Vibrio* spp. strains were cultured using thiosulfate citrate bile salt sucrose agar (TCBS agar, Eiken Chemical) at 35°C for overnight, and non-*Vibrio* strains were cultured using nutrient agar plate at 37°C for overnight.

According to the manufacturer's protocol, genomic DNA was extracted from all culture strains using the QIAamp DNA Mini Kit. Quantity of purified genomic template was measured

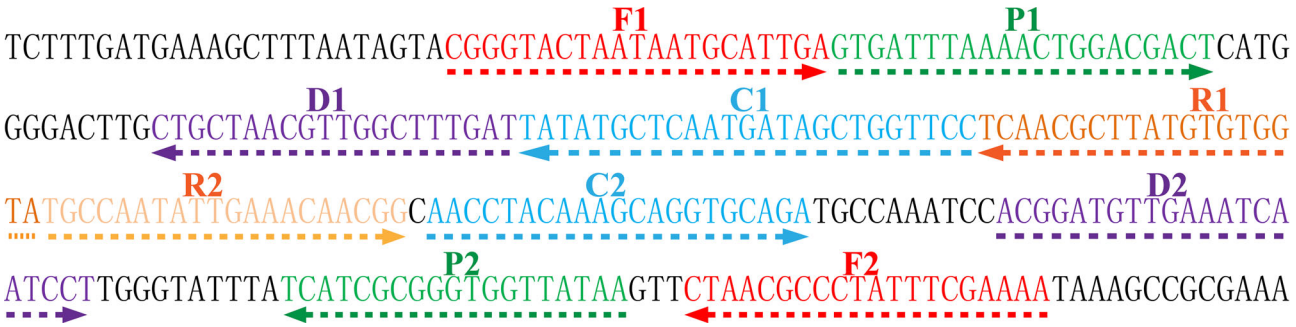

Figure 1. Sequence and location of *ompW* gene (*Vibrio cholerae*-specific gene) used to design multiple cross displacement amplification primers. The nucleotide sequence of the sense strand of *ompW* gene was shown. Right arrows and left arrows indicate sense and complementary sequences that were used.

Table 2. The primers used in this study.

Primers[a]	Sequences and modifications (5′-3′)	Length[b]	Gene
F1	CGGGTACTAATAATGCATTGA	21 nt	
CP1	GGAACCAGCTATCATTGAGCATATAGTGATTTAAAACTGGACGACT	46 mer	
CP1*	Biotin-GGAACCAGCTATCATTGAGCATATAGTGATTTAAAACTGGACGACT	46 mer	
C1	GGAACCAGCTATCATTGAGCATATA	25 nt	
C1*	FITC-GGAACCAGCTATCATTGAGCATATA	25 nt	
D1	ATCAAAGCCAACGTTAGCAG	20 nt	*ompW*
R1	TACCACACATAAGCGTTGA	19 nt	
R2	TGCCAATATTGAAACAACGG	20 nt	
D2	ACGGATGTTGAAATCAATCCT	21 nt	
C2	AACCTACAAAGCAGGTGCAGA	21 nt	
CP2	AACCTACAAAGCAGGTGCAGATTATAACCACCCGCGATGA	40 mer	
F2	TTTTCGAAATAGGGCGTTAG	20 nt	

CP1*, 5′-labeled with biotin when used in MCDA-LFB assay; C1*, 5′-labeled with FITC when used in MCDA-LFB assay.
[b] nt, nucleotide; mer, monomeric.

using a Nano drop ND-1000 instrument (Calibre, Beijing, China). An isolate of *V. cholerae* non-O1/non-O139/non-O141 designated as ICDC-NPVc001 (Table 1) was employed for the assay of optimization and sensitivity analysis with pure culture and spiked shrimp samples. The templates of *V. cholerae* ICDC-NPVc001 were serially diluted (10 ng, 10 pg, 10 fg, 1 fg and 0.1 fg per μl) for sensitivity examination of *V. cholerae*-MCDA-LFB detection and aliquots (1 μl) of each dilution were applied to the standard MCDA reaction.

Preparation of gold nanoparticle-based dipstick biosensor

The LFBs (4 × 6 cm) were prepared and assembled as previously described (Wang *et al.* 2016b, 2017a,b). Briefly, the sample pad, conjugate pad, NC membrane and absorbent pad were assembled on a plastic adhesive backing card. The capture reagents, including anti-FITC Ab (0.2 mg/mL) and biotin-BSA (2.5 mg/mL) in 0.01 M phosphated-buffered saline (PBS, PH 7.4), were sprayed onto NC membrane to form the test line (TL) and control line (CL). On the NC membrane, there are zones as TL (conjugated with anti-FITC) and CL (conjugated with anti-Dig), with each line separated by 5 mm. SA-G in 0.01M PBS (PH 7.4) was deposited on the conjugate pad of the strip. The assembled cards were cut into 4-mm wide strips (Deli No. 8012), and were then packaged in a plastic box. The biosensors were stored at the room temperature until use.

Design of MCDA assay primers

The MCDA primer pairs was designed based on the sequence of *ompW* coding region (GenBank accession no. X51948.1) using PRIMER PREMIER 5.0 and PrimerExplorer V4 (http://primerexplorer.jp/elamp4.0.0/index.html). A total of 10 primers, including two displacement primers (F1 and F2), two cross primer (CP1 and CP2) and six amplification primers (C1, C2, D1, D2, R1 and R2), were analyzed for hairpin structures and hybrids using the Integrated DNA Technologies design tools (http://www.idtdna.com/pages/scitools). Blast analysis suggested that the MCDA primer set was specific for *V. cholerae*. In order to detect MCDA products by LFB, the CP1 and C1 primers were 5′-labeled with biotin and fluorescein isothiocyanate (FITC), respectively. The details of primer positions, primer sequences and modifications of MCDA primers are exhibited in Fig. 1 and Table 2. All of the oligomers were synthesized and purified by ZiXi Biotech. Co., Ltd. (Beijing, China) at HPLC purification grade.

The MCDA assay

According to previous studies, the MCDA assays were carried using a 25-μl total reaction volume containing a mix of 0.4 μM each of displacement primers F1 and F2, 0.8 μM each of amplification primers C1* and C2, 1.2 μM each of amplification primers R1, R2, D1 and D2, 1.2 μM each of cross primers CP1 and CP1*, 2.4 μM cross primer CP2, 12.5 μl 2 × reaction mix (Loopamp DNA

amplification Kit), 1.25 μl of *Bst* DNA polymerase (10 U) and 1 μl DNA template (Wang *et al.* 2015, 2016b, 2017a,b). The amplification mixtures with 1 μl genomic template of *Vibrio parahaemolyticus* strain (*V. parahaemolyticus*, ICDC-VP001) and *Listeria monocytogenes* strain (*L. monocytogenes*, ATCC19114) were used as negative controls, and mixtures with 1 μl double distilled water were used as a blank control. The reaction temperature was optimized at varying temperatures (60°C–67°C with 1°C intervals) for 60 min, followed by heating at 85°C for 2 min to stop the reaction.

Four detection techniques, including turbidimeters (LA-320C), gel electrophoresis, colorimetric indicator (HNB) and LFB detection, were employed for detecting MCDA products. Real-time analysis of MCDA reactions was conducted using the Real-time Turbidimeter LA-320 by recording the optical density (OD) at 650 nm every 6 s. A positive reaction was defined as a threshold of >0.1. Reaction products (3 μl) were detected by 2% agarose gel electrophoresis; the specific ladder of multiple bands should be visible for positive reactions, but not in the negative and blank controls. When employing HNB reagent, the amplified products caused a color change from violet to sky blue, while the negative controls and blank control remained violet. Reaction mixtures (0.5 μl) were analyzed by LFB, and two red bands (TL and CL) should be seen in positive reactions. Only a visible red band (CL) was visual in negative and blank controls.

Sensitivity and specificity of *Vibrio cholerae*-MCDA-LFB assay

To determine the sensitivity of the MCDA-LFB method for detecting *V. cholerae*, the template DNA from ICDC-NPVc001 was serially diluted (10 ng, 10 pg, 10 fg, 1 fg and 0.1 fg per microliter). Then, the diluted templates were subjected to the MCDA-LFB assay in comparison with the PCR assay. The limit of detection (LoD) of MCDA-LFB and PCR assays was ascertained by genomic DNA amount of the template. Furthermore, detection by *V. cholerae*-MCDA-LFB was compared to that with a colorimetric indicator (HNB), 2% agarose gel electrophoresis and real-time turbidity. Three replicates of each dilution were examined.

The *ompW*-PCR assay was reported in a previous report, and the amplicon size was 588 bp (Nandi *et al.* 2000). Briefly, the PCR reaction was performed using a pair of primers specific for the *ompW* gene. The sequence of the forward primer was 5′-CACCAAGAAGGTGACTTTATTGTG-3′ and that of the reverse primer was 5′-GAACTTATAACCACCCGCG-3′. The following reaction conditions were used: 35 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 1 min followed by a final extension at 72°C for 5 min.

Specificity of *V. cholerae*-MCDA-LFB method was evaluated using DNA templates from 74 bacterial strains (Table 1). All samples were determined twice.

Optimization the amplification time of the *Vibrio cholerae*-MCDA-LFB assay

The MCDA mixture was incubated at the optimal temperature for different lengths of time (10 to 40 min at 10-min intervals). The amplification products were detected using an LFB, and two replicates of each amplification time were tested.

Vibrio cholerae-MCDA-LFB detection in shrimp samples

The shrimp samples (*Macrobrachium rosenbergii*) were obtained from a local market in Beijing, China. The shrimp samples (n = 18) were confirmed as being *V. cholera* negative according to the microbiological examination by enrichment in alkaline peptone water for overnight, and the shrimp homogenate was spread onto the TCBS agar. The yellow colonies were subjected to exact the DNA samples, and then the DNA templates were examined for the presence of *ompW* gene by PCR assay (Nandi *et al.* 2000). Only shrimp samples that were *V. cholera* negative were applied to the following spiked shrimp experiments.

Briefly, *V. cholerae* cultures were serially diluted (10^{-1} to 10^{-8}), and 100 μl of appropriate dilution (10^{-6}) were placed in triplicate on brain heart infusion. Colony forming units (CFUs) were counted after 24 h at 37°C. Then, 100 μl of diluted *V. cholerae* cultures (10^{-3} to 10^{-8}) were added to 900 μl of shrimp homogenates and mixed well. The number of *V. cholerae* was adjusted to ~4.1×10^5, 4.1×10^4, 4.1×10^3, 4.1×10^2, 4.1×10^1 and 4.1×10^0 CFU mL^{-1}. The spiked shrimp homogenate was centrifuged at 200 *g* for 5 min to remove tissue. The supernatant was transferred to a new microcentrifuge tube, and was centrifuged at 18 000 *g* for 5 min. After removal of the supernatant, the pellet was resuspended in phosphate buffer (20 μl), and then subjected to extract DNA samples. The extracted genomic templates were eluted in 20 μl of elution buffer. For the MCDA-LFB and PCR assays, 2 μl of the extracted DNA samples was employed as template and non-contaminated shrimp samples were served as negative control. The experiment was conducted in triplicate independently.

Vibrio cholerae-MCDA-LFB application for rapid diagnosis

In this report, the detection limit of *V. cholerae*-MCDA-LFB assay in combination with an enrichment step in shrimp samples was also evaluated. The artificial contaminative shrimp samples, which were 1000 mg shrimp homogenate containing ~2 CFUs of strain ICDC-NPVc001, were transferred into 5 mL of alkaline peptone water and homogenized, followed by incubation at 35°C for 0, 2, 4, 6, 8, 10, 12 and 24 h. Aliquots (1 mL) of the enrichment broth were subjected to extracted genomic DNA, and the extracted genomic templates were eluted in 20 μl of elution buffer. Aliquots (2 μl) of the extracted templates were used as a template in the MCDA-LFB method. The experiment was carried out twice independently.

RESULTS

Confirmation and detection of *Vibrio cholerae*-MCDA products

In order to examine the feasibility of *V. cholerae* MCDA primers, *V. cholerae* MCDA assays with the genomic templates from pure cultures were performed for 1 h at 63°C. The MCDA reactions were directly detected with naked eye by adding the colorimetric indicator (HNB) into amplification mixtures, and a positive amplification was indicated by a color change from violet to blue (Fig. 2A). After agarose gel electrophoresis, the positive MCDA reaction compared with negative and blank controls displayed the specific ladder of multiple bands (Fig. 2B). By LFB, two red bands, including TL and CL, appeared on the biosensors for the positive amplification, and single red band appeared on the biosensors for negative and blank controls (Fig. 2C). As shown in Fig. 2, the positive amplification occurred with templates from *V. cholerae* (ICDC-NPVc001), but not with the templates form *V. parahaemolyticus* (ICDC-VP001), *L. monocytogenes* (ATCC19114) and the blank control. Thus, these results indicated

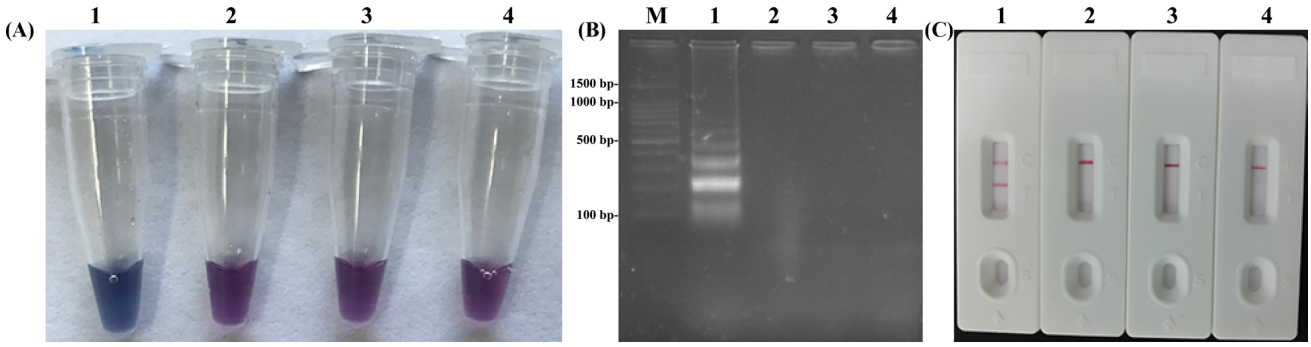

Figure 2. Confirmation and detection of *V. cholerae*-MCDA products. (**A**) By HNB, amplification products of *V. cholerae*-MCDA assay were visually analyzed by observation of the color change. (**B**) Agarose gel electrophoresis of *V. cholerae*-MCDA products was shown. (**C**) Lateral flow biosensor applied for visual detection of *V. cholerae*-MCDA products. Tube 1/lane 1/biosensor 1, positive amplification of *V. cholera* strain (ICDC-NPVc001); tube 2/lane 2/biosensor 2, negative control of *V. parahaemolyticus* strain (ICDC-VP001); tube 3/lane 3/biosensor 3, negative control of *L. monocytogenes* strain (ATCC19114); tube 4/lane 4/biosensor 4, blank control (double distilled water, DW). Lane M, DNA maker DL 100.

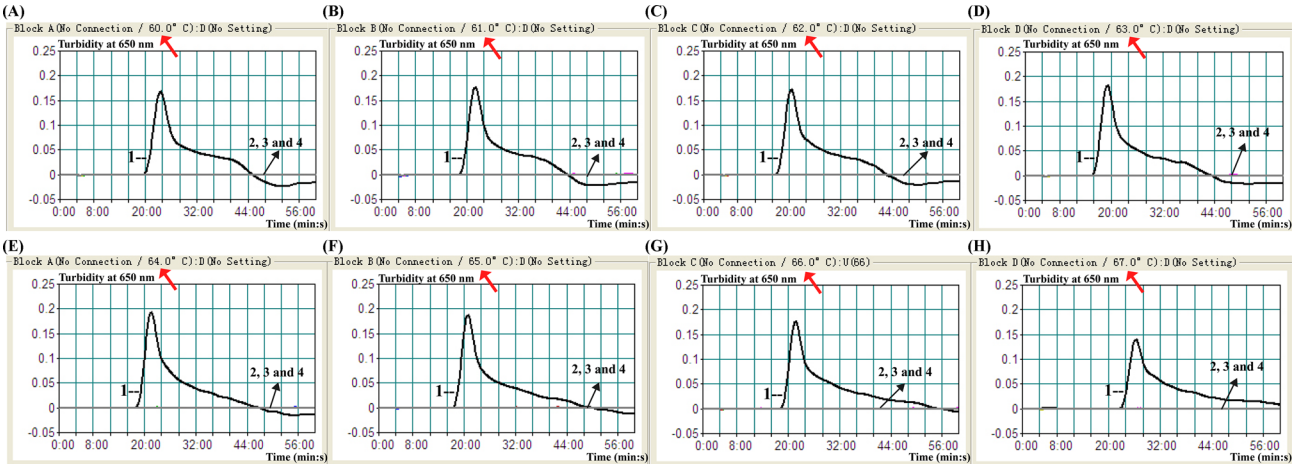

Figure 3. Optimal amplification temperature for *V. cholerae*-MCDA primer sets. The standard MCDA reactions for detection of *V. cholerae* were monitored by real-time measurement of turbidity, and the corresponding curves of concentrations of DNA were marked in the figures. The threshold value was 0.1, and the turbidity of >0.1 was considered to be positive. Eight kinetic graphs (**A**–**H**) were generated at various temperatures (60–67°C, 1°C intervals) with target pathogens DNA at the level of 10 pg per reaction. Signal 1, positive amplification of *V. cholera* strain (ICDC-NPVc001); signal 2, negative control of *V. parahaemolyticus* strain (ICDC-VP001); signal 3, negative control of *L. monocytogenes* strain (ATCC19114); signal 4, blank control (DW).

that the primer set was a good candidate for establishment of the MCDA-LFB method for *V. cholerae* detection.

Optimization of the temperature for *Vibrio cholerae*-MCDA assay

The optimal temperature for the *V. cholerae*-MCDA-LFB assay during the amplification stage was determined with *V. cholerae* genomic DNA (10 pg per reaction) at temperatures of 60°C–67°C for 1 h by monitoring turbidity. Although amplification targeting of *ompW* gene detected at all tested temperatures, a threshold value of absorbance (0.1) from the MCDA reaction was reached most quickly at 63°C (Fig. 3). After at least 1 h of incubation, no non-specific amplification was detected in the negative and blank controls. Herein, the reaction temperature of 63°C was used for the subsequent MCDA-LFB tests conducted in this study.

Specificity of *Vibrio cholerae*-MCDA-LFB assay

The specificity of *V. cholerae*-MCDA-LFB assay was verified with genomic templates (roughly 10 ng of DNA for each pathogen) extracted from 31 *V. cholerae* strains and 43 non-*V. cholerae* strains. The positive results were generated from all of the *V. cholerae* isolates, and two red lines (TL and CL) were appeared on the biosensors (Fig. 4). By contrast, only single red line (CL) appeared on the biosensor suggesting negative results for non-*V. cholerae* isolates and blank control.

Sensitivity of *Vibrio cholerae*-MCDA-LFB assay

In order to test the sensitivity of *V. cholerae*-MCDA-LFB assay, serial dilutions of the extracted DNA of *V. cholerae* (ICDC-NPVc001) were subjected to the MCDA-LFB method. The final concentration of templates was 10 ng, 10 pg, 10 fg, 1 fg and 0.1 fg per reaction mixture. As shown in Fig. 5, the experimental LoD of *V. cholerae*-MCDA-LFB approach was as few as 10 fg in pure culture. In addition, the sensitivity of the *V. cholerae*-MCDA-LFB was compared with those of MCDA detected products by real-time turbidity, colorimetric indicator (HNB) and 2% agarose gel electrophoresis. The results obtained using LFB were in conformity with other monitoring methods employed in this report (Fig. 5). In contrast, the *V. cholerae*-PCR assay had an LoD of 10 pg that

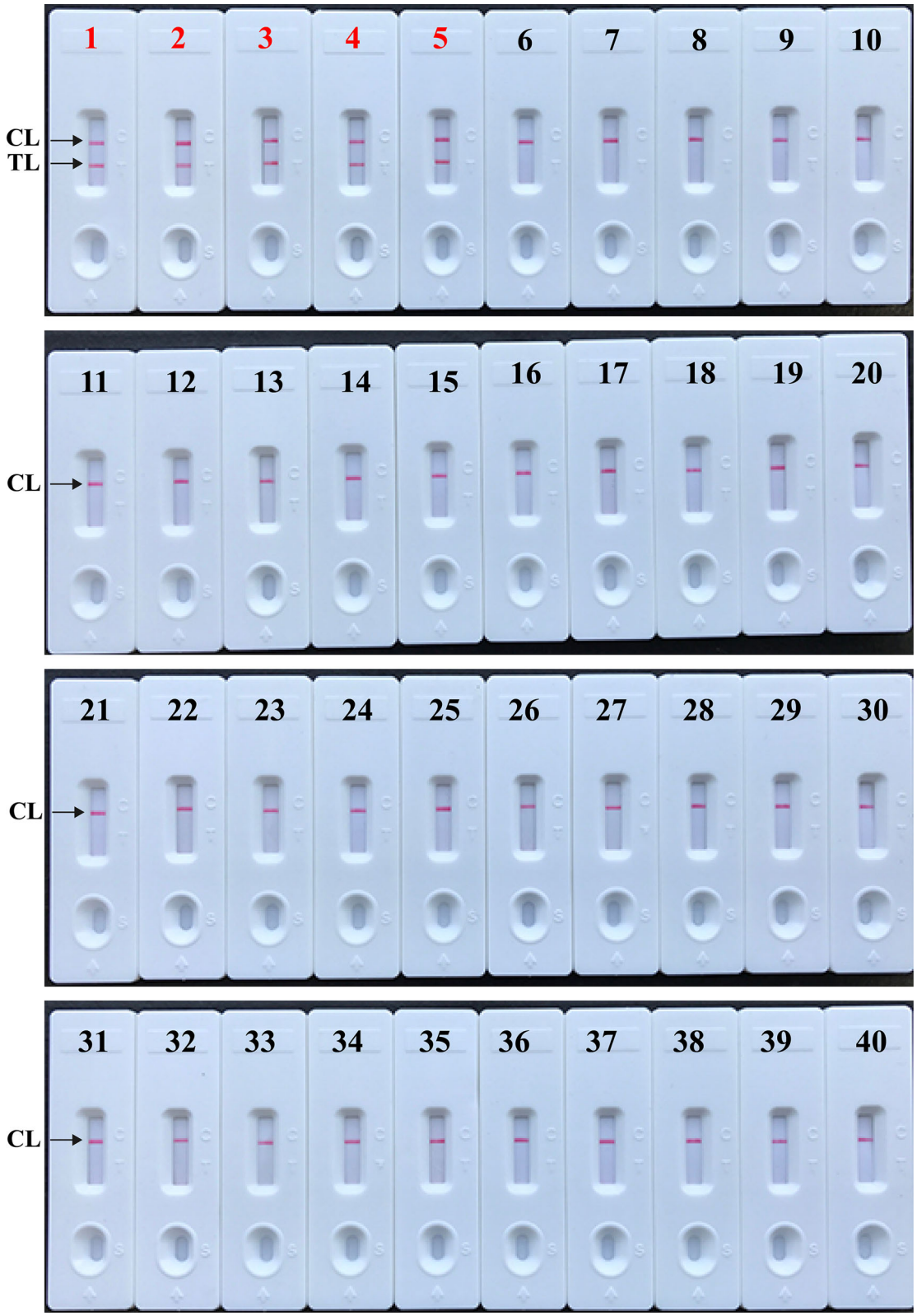

Figure 4. Specificity of *V. cholerae*-MCDA-LFB assay for distinct strains. The MCDA reactions were carried out using different genomic DNA templates and were analyzed by means of visual format. Biosensor 1, *V. cholerae* non-O1/non-O139 strain; biosensors 2–3, *V. cholerae* O1 strains; biosensors 4–5, *V. cholerae* O139 strains; biosensor 6, *V. parahaemolyticus* strain (ICDC-NVP001); biosensor 7, *V. vnlnificus* strain (ATCC27562); biosensor 8, isolated strain of *V. mimicus*; biosensor 9, isolated strain of *V. fluvialis*; biosensor 10, isolated strain of *V. alginolyticus*; biosensors 11–39, *L. monocytogenes*, *L. ivanovii*, *L. grayi*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, enteropathogenic *E. coli*, enterotoxigenic *E. coli*, enteroaggregative *E. coli*, enteroinvasive *E. coli*, enterohemorrhagic *E. coli*, *Plesiomonas shigelloides*, *Aeromonas hydrophila*, *Shigella dysenteriae*, *S. boydii*, *S. flexneria*, *S. sonneri*, *Salmonella*, *Enterococcus faecalis*, *Enterococcus faecium*, *Staphylococcus aureus*, *Staphylococcus epidemidis*, *Yersinia enterocolitica*, *Enterobacter cloacae*, *Bntorobater sakazakii*, *Bacillus cereus*, *Campylobacter jejuni*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, biosensor 40, blank control (DW).

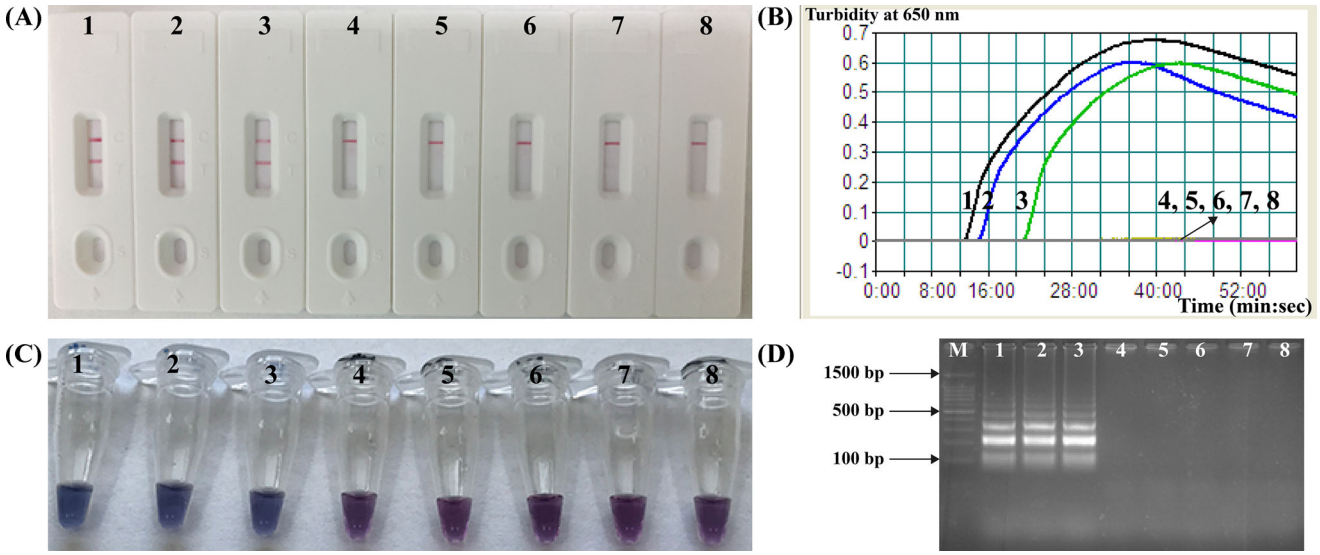

Figure 5. Sensitivity of MCDA-LFB assay using serially diluted genomic DNA with *V. cholerae* strain ICDC-NPVc001. Four measurement techniques, including later flow biosensor (**A**), real-time turbidity (**B**), HNB (**C**) and gel electrophoresis (**D**), were applied for detecting MCDA amplicons. The serial dilutions (10 ng, 10 pg, 10 fg, 1 fg and 0.1 fg) of target templates were subjected to standard MCDA reactions. Biosensors (A)/turbidity signals (B)/tubes (C)/lanes (D) 1–8 represented the DNA levels of 10 ng, 10 pg, 10 fg, 1 fg and 0.1 fg per reaction, negative control (10 pg of *V. parahaemolyticus* genomic DNA), negative control (10 pg of *L. monocytogenes* genomic DNA) and blank control (DW). The DNA levels of 10 ng, 10 pg and 10 fg per reaction yielded the positive reactions.

Table 3. Comparison of MCDA-LFB and PCR assays for detection of *V. cholerae* in pure culture and spiked shrimp samples.

Detection methods	LoD (no./reaction)	
	Pure culture	Spiked shrimp samples
MCDA-LFB	10 fg	4.1 CFU ~ (410 CFU mL^{-1})
PCR	10 pg	410 CFU ~ (41 000 CFU mL^{-1})

is 1000-fold less sensitive than that of the *V. cholerae*-MCDA-LFB assay (Fig. 5, Table 3).

Optimization of the time for *Vibrio cholerae*-MCDA-LFB assay

To evaluate the optimum duration of time require for the *V. cholerae*-MCDA-LFB assay during reaction stage, a total of four reaction times (10, 20, 30 and 40 min) were compared at 63°C according to the standard MCDA conditions. The lowest DNA level (10 fg of *V. cholerae* templates pre reaction) displayed two visible red lines (TL and CL) when the amplification only lasted for 30 min at 63°C (Fig. 6). Herein, the reaction time of 30 min was recommended as an optimal reaction time for *V. cholerae*-MCDA-LFB assay.

Application of MCDA-LFB to *Vibrio cholerae*-spiked shrimp homogenates

The *V. cholerae*-MCDA-LFB assay could generate positive results when the contaminated numbers of *V. cholerae* were more than 4.1 CFUs per reaction (~4.1×10^2 CFU mL^{-1}) (Fig. 7A). The *V. cholerae*-MCDA-LFB assay produced negative results at the concentrations lower than 0.41 CFUs per reaction (~4.1×10^1 CFU mL^{-1}), negative control and blank control. As observed with MCDA methods using genomic DNA from shrimp samples, analysis of the MCDA products was as sensitive with the biosensor approach with the other three techniques (Fig. 7). The sensitivity of *V. cholerae*-PCR assay was 410 CFUs per reaction or equivalent to 4.1×10^4 CFU mL^{-1} (Table 3). Based on these observations, the LoD of *V. cholerae*-MCDA-LFB assay was 100-fold more sensitive than that of *V. cholerae*-PCR method in *V. cholerae*-spiked shrimp homogenates.

Application of MCDA-LFB for rapid diagnosis

The capability of the MCDA-LFB method to rapidly diagnose low levels of target pathogen in *V. cholerae*-spiked shrimp homogenate samples was also examined. The typical MCDA-LFB judgment graph yielded from shrimp homogenate samples was displayed in Fig. 8A. None of 0- and 2-h enrichment samples tested positive for *V. cholerae* by the MCDA-LFB assay. The *V. cholerae*-MCDA-LFB method generated positive results for 4-, 6-, 8-, 10-, 12- and 24-h enrichment samples. As observed with MCDA methods using DNA from enrichment samples, detection of the MCDA products was as sensitive with the biosensor method with the real-time turbidity analysis (Fig. 8B).

DISCUSSION

Vibrio cholerae is an important human pathogen that is responsible for cholera, a severe acute watery diarrhea (Crowe *et al.* 2016). In the current study, a MCDA-LFB assay targeting the *ompW* gene was successfully established for the identification of all *V. cholerae* strains. In the MCDA-LFB assay, a set of 10 primers, which specially recognizes 10 regions of the target sequence, offers a high degree of specificity (Fig. 1). Moreover, the sequences of MCDA primer set were based on the species-specific gene (*ompW*), which was unique to *V. cholerae* (Nandi *et al.* 2000). The assay's specificity was successfully evaluated with the genomic templates extracted from 31 *V. cholerae* strains and 43 non-*V. cholerae* isolates, and the positive results were obtained from the assay of *V. cholerae* strains but not for non-*V. cholerae* and

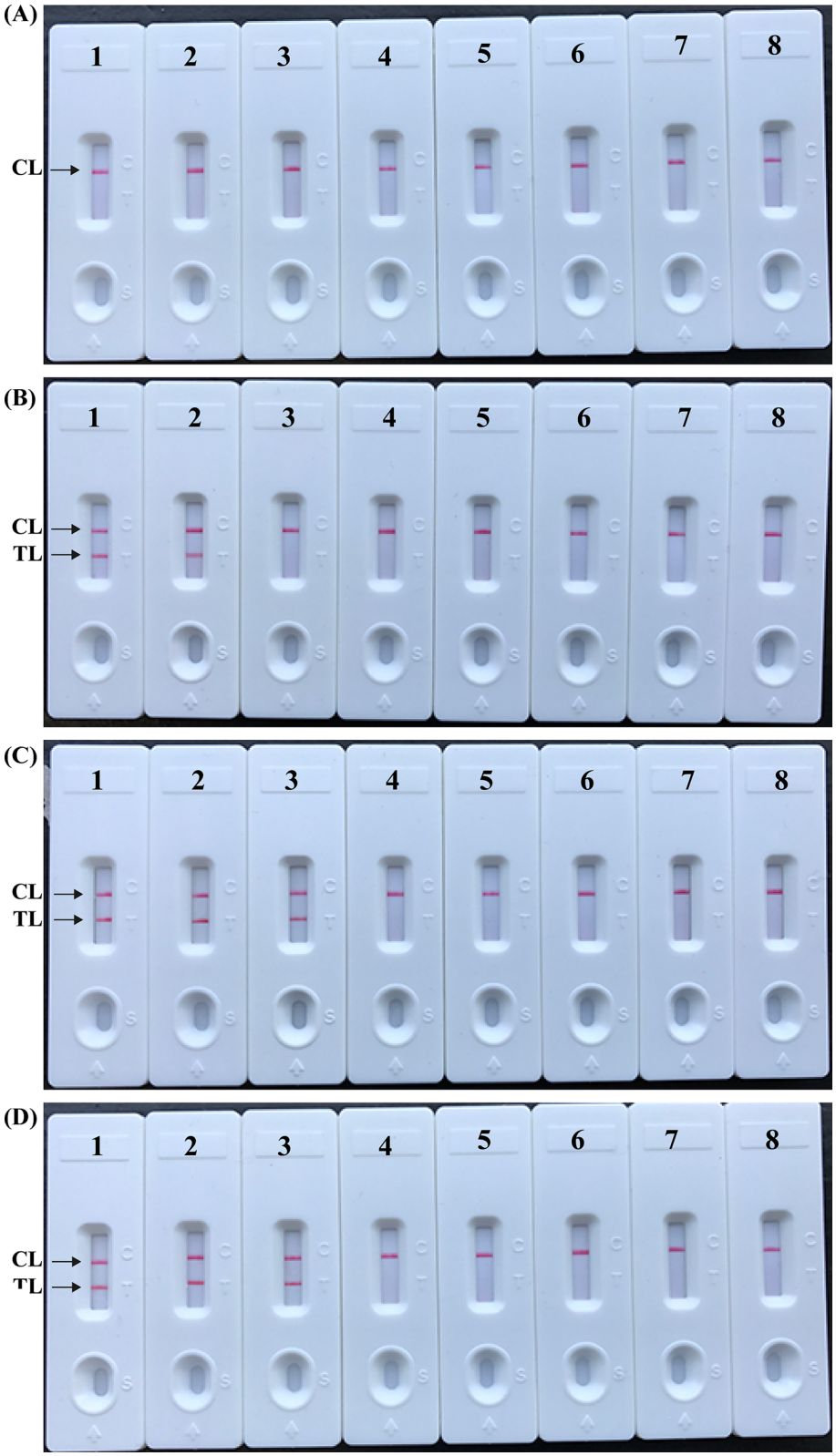

Figure 6. The optimal duration of time required for *V. cholerae*-MCDA-LFB method. Four distinct reaction times (**A**, 10 min; **B**, 20 min; **C**, 30 min; **D**, 40 min;) were examined and compared at 63°C. Biosensors 1, 2, 3, 4, 5, 6, 7 and 8 represent DNA levels of 10 ng of *V. cholerae* templates, 10 pg of *V. cholerae* templates, 10 fg of *V. cholerae* templates, 1 fg of *V. cholerae* templates, 0.1 fg *V. cholerae* templates per tube, negative control (*V. parahaemolyticus*, 10 pg per reaciton), negative control (*L. monocytogenes*, 10 pg per reaction) and blank control (DW). The best sensitivity was seen when the reaction lasted for 30 min (C). TL, test line; CL, control line.

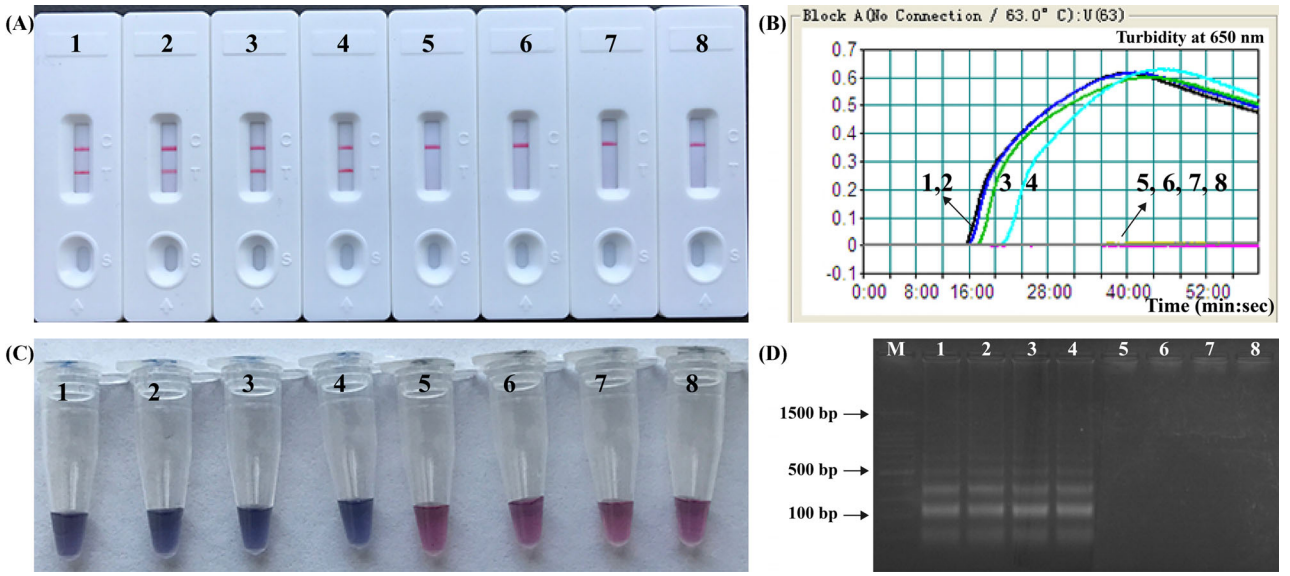

Figure 7. Sensitivity of MCDA-LFB assay for detecting *V. cholerae* in spiked shrimp samples. Four measurement techniques, including later flow biosensor (**A**), real-time turbidity (**B**), colorimetric indicator (HNB) (**C**) and gel electrophoresis (**D**), were applied for detecting MCDA amplicons. The serial dilutions of target templates were subjected to standard MCDA reactions. Strips (A)/turbidity signals (B)/tubes (C)/lanes (D) 1–8 represented the DNA levels of 4100 CFU, 410 CFU, 41 CFU, 4.1 CFU, 0.41 CFU and 0.041 CFU per reaction, negative control (non-spiked shrimp sample) and blank control (DW). The genomic DNA levels of 4100 CFU, 410 CFU, 41 CFU and 4.1 CFU per reaction yielded the positive reactions.

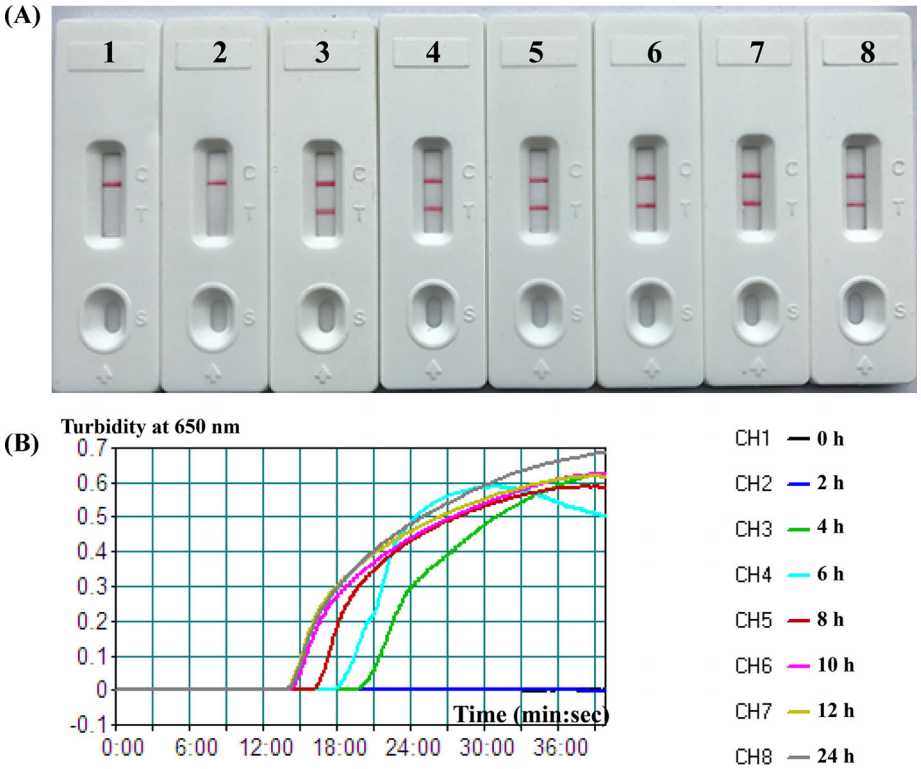

Figure 8. The *V. cholerae*-MCDA-LFB detection graph obtained when testing shrimp samples spiked with the low level of *V. cholerae* strains after various enrichment periods in alkaline peptone water. Biosensors 1–8 (CH1–8), 0, 2, 4, 6, 8, 10, 12 and 24 h. CH, channel.

blank control (Fig. 4). Hence, the presented method was highly specific to *V. cholerae* over other non-*V. cholerae* strains, which could be a valuable tool for identifying target pathogen with high selectivity.

Compared to PCR-based methodologies, MCDA-LFB assay do not require the thermal denature and the changes in temperatures, eliminating the use of complicated and precise equipment. The MCDA-LFB method only needs a simple incubation at 63°C, and thus facilitates its application in resource-poor settings (Wang *et al.* 2015). Importantly, the whole procedure, including sample processing (30 min), MCDA amplification (30 min) and results reporting (2 min), could be finished within

65 min, at least 2 h less than the standard *V. cholerae*-PCR method. Herein, this assay exhibited distinct advantages in terms of equipment requirements and detection time.

Generally, the MCDA amplified products are detected by naked eye for the presence of a white precipitate generated from magnesium pyrophosphate, by visualization of the products under natural light after adding colorimetric indicator (HNB or FD), or under UV irradiation after adding SYBR Green I or calcein dyes, by the use of spectrophotometric instrument to measure turbidity or by agarose gel electrophoresis (Zhang, Lowe and Gooding 2014; Wang *et al*. 2017a,b). First, because of the use of a set of 10 primers, MCDA yields a complex mixture of nucleic acid products; thus, these product analysis techniques cannot differentiate between specific amplicons and non-specific amplicons (Zhang, Lowe and Gooding 2014). Second, the assessment of color or turbidity with naked eye is potentially subjective, and there is the strong possibility that a sample may be somewhat ambiguous to the unaided eye when the concentration of target templates is very low. Third, these monitoring techniques required an additional analysis procedure (gel electrophoresis) or turbidimeter (turbidity detection), and the resultant instrumental restraint can limit the uptake of MCDA assay in field and point-of-care settings. In the present report, we used a simple biosensor (LFB) housed in a plastic device to objectively and visually detect MCDA products within 2 min. Hence, the *V. cholerae*-MCDA-LFB approach provides a rapid, simple and nearly instrument-free platform for detection of target bacterium with easily interpretable results.

In this report, by comparing the sensitivity of MCDA-LFB and conventional PCR, we found that the LoD of *V. cholerae*-MCDA-LFB assay was 10 fg per reaction in pure culture, which is 1000-fold more sensitive than that of *V. cholerae*-PCR method (Fig. 5, Table 3). MCDA-LFB was also a detection technique with better sensitivity when it was applied in shrimp samples (Fig. 7, Table 3). The sensitivity of *V. cholerae*-MCDA-LFB was 4.1 CFUs per reaction in spiked homogenates, which is 100-fold more sensitive than that of *V. cholerae*-PCR method. The poor sensitivity of traditional PCR in spiked shrimp sample may be due to inhibitors in shrimp homogenates. The previous reports indicated that isothermal amplification technique was less vulnerable to certain inhibitors present in the various food contexts (Kaneko *et al*. 2007; Wang *et al*. 2014).

In conclusion, the MCDA-LFB assay for detection of *V. cholerae* based on *ompW* gene was successfully devised. This method presented in the report display high selectivity for target pathogen detection, and had the sensitivity of 10 fg per reaction with pure culture and 4.1 CFUs per reaction with spiked shrimp homogenates. Comparing with culture- and PCR-based methods, the MCDA-LFB protocol was more convenient, rapid, simple, sensitive and does not costly specialized equipment. Thus, the MCDA-LFB assay will hopefully offer a strategy for rapid detection of the pathogenic bacterium, which is especially suitable for application in the field and resource-limited situations.

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Conflict of interest. None declared.

Disclosure

YW and CY have filed for a patent from the State Intellectual Property Office of China, which covers the novel technique and sequences included in this manuscript (application number: 201710323212.7).

REFERENCES

Chatterjee S, Ghosh K, Raychoudhuri A *et al*. Incidence, virulence factors, and clonality among clinical strains of non-O1, non-O139 *Vibrio cholerae* isolates from hospitalized diarrheal patients in Kolkata, India. *J Clin Microbiol* 2009;**47**:1087–95.

Chen W, Zhang J, Lu G *et al*. Development of an immunochromatographic lateral flow device for rapid diagnosis of *Vibrio cholerae* O1 serotype Ogawa. *Clin Biochem* 2014;**47**:448–54.

Crowe SJ, Newton AE, Gould LH *et al*. Vibriosis, not cholera: toxigenic *Vibrio cholerae* non-O1, non-O139 infections in the United States, 1984–2014. *Epidemiol Infect* 2016;**144**:3335–41.

Dick MH, Guillerm M, Moussy F *et al*. Review of two decades of cholera diagnostics–how far have we really come? *PLoS Neglect Trop D* 2012;**6**:e1845.

Fraser B. Haiti still gripped by cholera as election looms. *The Lancet* 2010;**376**:1813–4.

Kaneko H, Kawana T, Fukushima E *et al*. Tolerance of loop-mediated isothermal amplification to a culture medium and biological substances. *J Biochem Bioph Meth* 2007;**70**:499–501.

Kirigia JM, Sambo LG, Yokouide A *et al*. Economic burden of cholera in the WHO African region. *BMC Int Health Hum R* 2009;**9**:8.

Marin MA, Thompson CC, Freitas FS *et al*. Cholera outbreaks in Nigeria are associated with multidrug resistant atypical El Tor and non-O1/non-O139 *Vibrio cholerae*. *PLoS Neglect Trop D* 2013;**7**:e2049.

Nandi B, Nandy RK, Mukhopadhyay S *et al*. Rapid method for species-specific identification of *Vibrio cholerae* using primers targeted to the gene of outer membrane protein OmpW. *J Clin Microbiol* 2000;**38**:4145–51.

O'Hara CM, Sowers EG, Bopp CA *et al*. Accuracy of six commercially available systems for identification of members of the family vibrionaceae. *J Clin Microbiol* 2003;**41**:5654–9.

Patra T, Chatterjee S, Raychoudhuri A *et al*. Emergence and progression of Vibrio cholerae O1 El Tor variants and progenitor strains of Mozambique variants in Kolkata, India. *Int J Med Microbiol* 2011;**301**:310–7.

Sack DA, Sack RB, Nair GB *et al*. Cholera. *Lancet* 2004;**363**:223–33.

Sack RB, Siddique AK, Longini IM, Jr *et al*. A 4-year study of the epidemiology of Vibrio cholerae in four rural areas of Bangladesh. *J Infect Dis* 2003;**187**:96–101.

Thompson FL, Swings J. Taxonomy of the vibrios[M]//The biology of vibrios. American Society of Microbiology, 2006, 29–43.

Wang Y, Wang Y, Ma A *et al*. Rapid and sensitive detection of *Listeria monocytogenes* by cross-priming amplification of lmo0733 gene. *FEMS Microbiol Lett* 2014;**361**:43–51.

Wang Y, Wang Y, Xu J *et al*. Development of multiple cross displacement amplification label-based gold nanoparticles

lateral flow biosensor for detection of *Shigella* spp[J]. *Front Microbiol* 2016a;**7**.

Wang Y, Li H, Li D *et al*. Multiple cross displacement amplification combined with gold nanoparticle-based lateral flow biosensor for detection of *Vibrio parahaemolyticus*. *Front Microbiol* 2016b;**7**.

Wang Y, Li H, Wang Y *et al*. Development of multiple cross displacement amplification label-based gold nanoparticles lateral flow biosensor for detection of *Listeria monocytogenes*. *Int J Nanomed* 2017a;**12**:473.

Wang Y, Wang Y, Ma A-J *et al*. Rapid and sensitive isothermal detection of nucleic-acid sequence by multiple cross displacement amplification. *Sci Rep* 2015;**5**:11902.

Wang Y, Wang Y, Zhang L *et al*. Visual and multiplex detection of nucleic acid sequence by multiple cross displacement amplification coupled with gold nanoparticle-based lateral flow biosensor. *Sensors Actuat B: Chem* 2017b;**241**:1283–93.

Zhang X, Lowe SB, Gooding JJ. Brief review of monitoring methods for loop-mediated isothermal amplification (LAMP). *Biosens Bioelectron* 2014;**61**:491–9.