Rapid and sensitive detection of *Plesiomonas shigelloides* by cross-priming amplification of the *hugA* gene

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Abstract. *Plesiomonas shigelloides (P. shigelloides)* is implicated as an aetiological agent of human gastroenteritis in humans, for which reliable laboratory detection of *P. shigelloides* is clinically and epidemiologically desirable. A simple molecular method for rapid detection of *P. shigelloides* using cross-priming amplification (CPA) has been developed, with *hugA* as the target. The *hugA* gene is required for haem iron utilisation and is critical for the survival and growth of *P. shigelloides*. The assay output was visualised as a colour change with no need to open the reaction tubes, and no false-positive results were detected for the 33 non- *P. shigelloides* strains examined to assess assay specificity. The limit of detection was 200 fg *P. shigelloides* DNA per reaction and $3x10^3$ CFU per g in human stools, which was 100 and 10-fold more sensitive than polymerase chain reaction, respectively. The CPA method was used to detect the presence of *P. shigelloides* in stool specimens from 70 patients with diarrhoea and 30 environmental water samples, with no difference in accuracy between the CPA assay and the biological culture. The present study, therefore, suggests that the *P. shigelloides hugA* CPA assay may represent a valuable tool for rapid and sensitive detection of *P. shigelloides* in primary care facilities and clinical laboratories.

Introduction

Plesiomonas shigelloides (*P. shigelloides*) is the only species member of the *Plesiomonas* genus, and the only oxidase-positive member of the *Enterobacteriaceae* family (1). *P. shigelloides* is present worldwide, primarily in aquatic environments, including freshwater, estuarine and marine environments (2-4). *P. shigelloides* induces various types of gastroenteritis, including acute secretory gastroenteritis, invasive shigellosis-like disease and cholera-like illness, infections increasing in recent years (5-7).

In humans, although extra-intestinal diseases of *P. shigelloides* are rare, it has been associated with secondary infections in immunocompromised states, including malignancy, blood disorders and hepatobiliary disease (8-10). *P. shigelloides* may often be missed in stool samples due to its small colony size and relatively low prevalence in gastrointestinal samples. The lack of a routine assay for *P. shigelloides* in cases of gastroenteritis means that this bacterium is identified only occasionally (11). Xia *et al* (12) reported a case of meningoencephalitis caused by *P. shigelloides* with a fatal outcome in a Chinese neonate. Therefore, its earlier and accurate identification, and the prescription of the correct antibiotic therapy may be critical for patient prognosis.

The traditional culture-based approaches for detection of *P. shigelloides* involve enrichment in liquid media and isolation of colonies on selective media. Although extensively used, these assays are time-consuming and laborious, taking more than three days. In addition, the isolates of *P. shigelloides* appear as green colonies on Hektoen enteric agar, with an appearance similar to that of *Shigella* (13).

Molecular-based techniques, including polymerase chain reaction (PCR) and quantitative PCR assays, have been established for the detection of *P. shigelloides*, producing reliable results. However, PCR-based techniques rely on expensive thermal cycler or complex sample-handling procedures, limiting its application (14). Therefore, there is an urgent requirement to devise a novel strategy for rapid, robust and sensitive identification of *P. shigelloides* using simple equipment.

Loop-mediated isothermal amplification (LAMP), as a rapid, specific and sensitive detection methodology, has been used to detect various pathogens, including parasites, fungi, bacteria and viruses (15). However, primer design for LAMP techniques is complicated, requiring a specific, long, highly conserved fragment. This limits the application of LAMP for the detection of pathogens (16). A novel technology, cross-priming

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amplification (CPA), overcomes the technical difficulties posed by current LAMP approaches, which contains five specially designed primers (1s, 2a, 3a, 4s and 5a) that recognise five conserved regions on the target sequence. Each cross primer contains 5' tail sequences identical to each other's priming site and thus introduces additional priming sites in each round of extension. The primers are designed to accomplish the basic goal of isothermal generation of single-stranded DNA (ssDNA) using a strand-displacing polymerase such as *Bst*, and the DNA target sequence may be amplified without an initial denaturation step or addition of a nicking enzyme (17-20). The CPA products may be detected by an increase in turbidity, agarose gel electrophoresis of amplicons or by visualization of a colour alteration in the presence of Loopamp® Fluorescent Detection Reagent.

Several potential virulence factors of *P. shigelloides* have been described, however, the pathogenesis of *P. shigelloides*-associated gastroenteritis remains to be elucidated (21,22). Acquisition of iron has been demonstrated to be involved in the virulence of a variety of bacterial pathogens (23,24). Heme is the primary source of iron within the body, and numerous pathogenic bacteria carry heme transport systems (25). The strains of *P. shigelloides* express highly specific outer membrane receptors that bind, extract and transport heme into the bacterial periplasm (26). The *hugA* genes (heme iron utilization locuaccession no. AY008342.1) encoding the heme iron utilization system of P. shigelloides have been isolated and characterized, and are essential for the growth of *P. shigelloides*.

The present study aimed to develop a rapid, cost-effective and efficient CPA method for detecting *P. shigelloides*, and evaluating the assay performance with pathogen-simulated human stool. In addition, the CPA method was compared with PCR to determine the sensitivity and evaluate the practical application in clinical samples.

Materials and methods

Ethics statement. Stool specimens were acquired from 70 patients with diarrhoea, aged from 18 to 50 years old, and written informed consent was obtained from all participants. The study was reviewed and approved by the ethics committee of the National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention (Beijing, China), according to the medical research regulations of the Ministry of Health (Beijing, China; approval no. ICDC-2014003).

Bacterial strains. A total of 53 strains (20 *P. shigelloides* strains and 33 non-*P. shigelloides* strains, listed in Table I) were used for specificity testing. The bacterial load of the strains used for specificity evaluation was 10^5 pg/ml, which is high enough to prevent false-negative amplifications. *P. shigelloides* ATCC 51903 (GenBank accession number AY008342.1) was selected as the positive control for the assay optimisation, sensitivity evaluation, and to spike human stool samples. All strains were cultured overnight at 37˚C on brain heart infusion (BHI) agar (BD Biosciences, Franklin Lakes, NJ, USA).

Genomic DNA extraction. Bacterial genomic DNA was extracted from all cultured strains using DNA extraction kits (QIAamp DNA minikitQiagen, Hilden, Germany) according to the manufacturer's instructions.

P. shigelloides hugA CPA primers and reaction conditions. A set of five primers was manually designed to target the nucleotide sequence of *P. shigelloides* ATCC 51903, based on the mechanism of CPA (27). The sequences and locations of the primers within *hugA* are presented in Table II and Fig. 1. CPA reactions were performed using the Loopamp kit (Eiken Chemical Co., Ltd., Tokyo, Japan) in a final volume of 20 μl containing 2.4 mM cross primer As, 1.44 mM each of primers 2a and 3a, 0.3 mM each of displacement primers 4s and 5a, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 4 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 0.8 M betaine, 1.4 mM deoxynucleoside triphosphates (dNTPs), 1 μl of *Bst* DNA polymerase (8 U μl^{-1}), 1 μl Loopamp Fluorescent Detection Reagent (Eiken Chemical Co., Ltd.) and 1 μl DNA template. The reaction mixture was incubated in an LA320 Real-Time Turbidimeter (Teramecs Co., Ltd., Kyoto, Japan) at 63˚C for 60 min, and then heated at 95˚C for 5 min to terminate the reaction. Amplified products were directly detected by observing a colour change from orange to green by the naked eye, or by electrophoresis on 2% agarose gels using staining with GoldenView reagent. Furthermore, real-time monitoring of the CPA reaction was performed by recording the optical density at 650 nm every 6 sec using the LA-320C Real-Time Turbidimeter. A positive reaction was defined as a turbidity cut-off value of >0.1 within 60 min.

Evaluation of the specificity, sensitivity and reproducibility of the P. shigelloides hugA CPA assay. To determine the specificity of the CPA assay, the CPA reaction was performed under the conditions described above with DNA templates from 20 *P. shigelloides* and 33 non-*P. shigelloides* strains (Table I). All detection assays were performed in triplicate.

To assess the analytical sensitivity of CPA assay, CPA assays were performed using serial dilutions (20, 2 ng, 200, 20, 2 pg, 200, 100 and 50 fg per μl) of *P. shigelloides* genomic DNA. The genomic templates (1 μl) were added into the CPA mixture and at least 3 replicates of each dilution were assessed to define the limit of detection (LoD) of the CPA approach. Mixtures without DNA templates served as a negative control. The sensitivity of the CPA assay on *P. shigelloides* was determined by analyzing the amplifications produced from the serial dilutions of the *P. shigelloides* genomic DNA.

To compare the sensitivities of the CPA and PCR assay in pure culture, template DNA from *P. shigelloides* (ATCC 51903) was serially diluted (20, 2.0 ng, 200, 20, 2.0 pg, 200 fg, 100 and 50 fg per μl). The LoD of CPA and PCR was ascertained using the two assays.

To evaluate the reproducibility of the CPA assay, different concentrations (20 ng, 200 and 2.0 pg) of template DNA from *P. shigelloides* (ATCC 51903) were amplified two ways (10 times on one day and once each on 10 different days). The intra-assay and inter-assay variation were analysed at the time of precipitation, as measured by turbidity on the Real-Time Turbidimeter. The coefficient of variation (CV) is equal to the standard deviation (SD) divided by the mean average, multiplied by 100. Statistical analyses were conducted using SPSS software (version, 19.0; IBM SPSS, Armonk, NY, USA).

Table I. Bacterial strains used in the present study.

Latin name	Strain no. (source of strain)	No. of strains
Plesiomonas shigelloides	ATCC 51903	1
	Isolated strains (ICDC)	19
Enteropathogenic *Escherichia coli*	Isolated strain (ICDC)	1
Enterotoxigenic *Escherichia coli*	Isolated strain (ICDC)	1
Enteroinvasive *Escherichia coli*	Isolated strain (ICDC)	1
Enterohaemorrhagic *Escherichia coli*	EDL 933 (isolated previously in our laboratory)	1
Enteroaggregative *Escherichia coli*	Isolated strain (ICDC)	1
Salmonella enterica	ATCC 14028	1
Shigella flexneri	Isolated strain (ICDC)	1
Shigella sonnei	ATCC 25931	1
Proteus vulgaris	Isolated strain (ICDC)	1
Aeromonas veronii	ATCC 35622	1
Aeromonas salmonicida	ATCC 7965	1
Aeromonas caviae	ATCC 15468	1
Aeromonas media	ATCC 33907	1
Clostridium perfringens	Isolated strain (ICDC)	1
Enterobacter cloacae	Isolated strain (ICDC)	1
Serratia marcescens	Isolated strain (ICDC)	1
Vibrio parahaemolyticus	ATCC 17802	1
Staphylococcus aureus	ATCC 6538	1
Streptococcus pneumoniae	Isolated strain (ICDC)	1
Streptococcus pyogenes	Isolated strain (ICDC)	1
Streptococcus sanguis	Isolated strain (ICDC)	1
Streptococcus salivarius	Isolated strain (ICDC)	1
Streptococcus bovis	Isolated strain (ICDC)	1
Enterococcus faecalis	ATCC 35667	1
Yersinia enterocolitica	ATCC 23715	1
Pseudomonas aeruginosa	ATCC 15442	1
Aeromonas hydrophila	ATCC 7966	1
Listeria monocytogenes	ATCC 54003	2
Enterobacter sakazakii	ATCC 51329	1
Campylobacter jejuni	ATCC 33291	1
Vibrio minicus	Isolated strain (ICDC)	1
Vibrio vulnificus	Isolated strain (ICDC)	1

ATCC, American type culture collection; ICDC, national institute for communicable disease control and prevention, Chinese center for disease control and prevention.

PCR amplifications were performed in a final volume of 20 μl containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.001% gelatin, 1.5 mM $MgCl_2$, 0.2 μM each of hugA forward and hugA reverse primers, 0.2 mM each of dNTPs, 0.5 units of *Ex Taq* DNA polymerase (Takara Bio, Inc., Otsu, Japan) and 1 μl DNA template. The program consisted of an initial denaturation step of 5 min at 95˚C, 35 cycles of 30 sec at 95˚C, 30 sec at 60˚C, and 30 sec at 72˚C, and a final 5 min extension at 72˚C. The PCR products were visualised by 2% agarose gel electrophoresis to verify the presence of the expected 435-bp fragment.

P. shigelloides hugA CPA application in simulated human stools specimens. Human stool specimens were obtained from a healthy donor with the written informed consent. The human stool specimens were confirmed to be *P. shigelloides*-negative using a traditional culture assay and PCR amplification (Table III). To determine the LoD of CPA in human stool, 10-fold serial dilutions of a mid-log phase culture of *P. shigelloides* grown in BHI broth at 37˚C were prepared in PBS, quantified using the standard plating method, and added to the stool samples at $3x10^1$-$3x10^6$ CFU/g. Aliquots (0.2 g) of the stools were used for DNA extraction with a QIAamp DNA Mini kit. This experiment was performed in triplicate independently, and the supernatants (2 μl) were used for CPA and PCR.

Practical application of the P. shigelloides hugA CPA assay. To estimate the feasibility of the CPA assay to detect *P. shigelloides* in clinical samples, 100 samples (70 stool specimens

Table II. CPA and PCR primers used to detect *Plesiomonas shigelloides*.

Assay type	Primer/probe name	Sequence (5'-3')	Length (nt)
hugA-CPA	AS (2a+1s)	AAGCCAATCCCGATTTGAAACCTTTTG TTAAAGCGCATCTGAGCTGAG	48
	3a	GGAGACCGCCAAAAACAAAGAGAT	24
	2a	AAGCCAATCCCGATTTGAAACC	22
	4s	GTCGCCCCAAACGCTAACTC	20
	5a	CGATGGGCTGCAACGTGTT	19
hugA-PCR	F	GCGAGCGGGAAGGGAAGAACC	21
	R	GTCGCCCCAAACGCTAACTCATCA	24

CPA, cross-priming amplification; PCR, polymerase chain reaction; F, forward; R, reverse.

Table III. Reproducibility of the *Plesiomonas shigelloides hugA* cross-priming amplification assay.

Reproducibility	Template DNA (pg/reaction)	Number of detections	Mean time of precipitation (mins)	Standard deviation	Coefficient of variation (%)
Intra-assay	$2x10^4$	10	23.4	0.21	0.90
	$2x10^2$	10	27.3	0.37	1.36
	2	10	38.4	0.49	1.28
Inter-assay	$2x10^4$	10	23.3	0.23	0.99
	$2x10^2$	10	27.5	0.33	1.20
	2	10	38.7	0.66	1.71

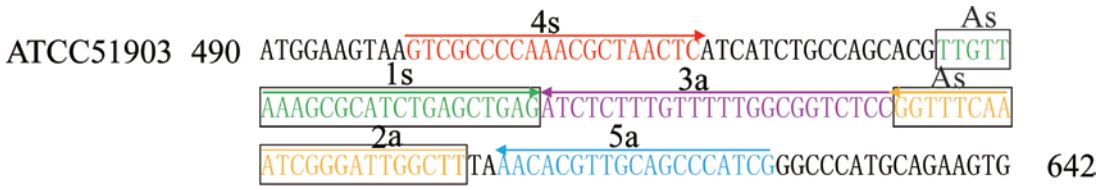

Figure 1. Target sequence and primer locations. Nucleotide sequence of the *hugA* gene fragment from *Plesiomonas shigelloides* ATCC 51903 used to design the cross-priming amplification primers. The locations of primers within the *hugA* gene are shown as right and left arrows indicating whether the sense or complementary sequence was used.

from patients with diarrhoea and 30 water samples from the environment) were analysed using the CPA method, and compared with the results from the traditional culture and PCR methods. Culture-based detection of stool samples was performed by enriching 2 g stool specimens in 20 ml tetrathionate broth without iodine (Oxoid; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 10 h at 37˚C, and then streaking on inositol brilliant green bile salts (IBB) agar (Oxoid; Thermo Fisher Scientific, Inc.) and the plates incubated at 35˚C for 24 h. Pink colonies suspected to be *P. shigelloides* were Gram stained, picked onto BHI agar at 37˚C for 18 h and subjected to biochemical tests using the API 20E system (BioMérieux, Marcy-l'Étoile, France) (25).

Water samples (500 ml) were filtered through sterile analytical filters (NalgenThermo Fisher Scientific, Inc.) with pore sizes of 0.45 μm, within 30 h of sample collection. The filters were enriched in 20 ml tetrathionate broth without iodine for 10 h at 37˚C, streaked on IBB agar and the plates were incubated at 35˚C for 24 h (25). Pink colonies suspected to be *P. shigelloides* were Gram stained, picked onto BHI agar at 37˚C for 18 h and subjected to biochemical tests using the API 20E system.

DNA was extracted from 1 ml aliquots of the enrichment broth using the QIAamp DNA Mini kit, and 2 μl of each DNA extract was used as the template in the CPA and PCR assays. *P. shigelloides* (ATCC 51903) genomic DNA was used as the positive control template, and sterile water was used as the negative control template.

Results

Primer design for the P. shigelloides hugA CPA assay. For the *P. shigelloides*-specific *hugA* gene, a set of 5 primers, which targeted 5 distinct regions, was designed for the CPA assay by sequence alignment and primer software Primer Premier 5.0

(Premier Biosoft International, Palo Alto, CA, USA). These included the amplification primer 2a and 1s, designated as the cross primer (As) and two amplification primers (3a and 2a). The specificity of the CPA primers was confirmed using the NCBI Basic Local Alignment Search Tool (National Institutes of Health, Bethesda, MD, USA). The details of the primers are presented in Table II and Fig. 1.

Confirmation and detection of P. shigelloides CPA products. The amplification products were examined by visual inspection using Loopamp Fluorescent Detection Reagent and the positive amplifications were directly observed due to the colour change from the original orange to green (Fig. 2A). In addition, the conventional CPA products were assessed by 2% agarose gel electrophoresis, and positive results demonstrated a typical ladder-like pattern (Fig. 2B).

Specificity of the P. shigelloides hugA CPA assay. The specificity of the CPA assay towards the *P. shigelloides hugA* gene was examined by performing the assay with DNA from 53 bacterial strains from 29 different species as the template (Table I). The 20 *P. shigelloides* strains were correctly identified, whereas no amplification was observed in the 33 non-*P. shigelloides* strains. The results demonstrated that the specificity of the CPA assay was 100%, and the sequence revealed no cross-reaction with different pathogens.

Sensitivity of the P. shigelloides hugA CPA assay. The sensitivity of CPA assay towards *P. shigelloides* was examined by determining the LoD of reactions performed with serial dilutions of *P. shigelloides* genomic DNA (20, 2 ng, 200, 20, 2 pg, 200, 100 and 50 fg per μl). The LoD of CPA (Fig. 3A) was 200 fg DNA/tube, whereas the LoD of PCR assay was 20 pg DNA/tube (Fig. 3B). These results indicated that the CPA assay was 100-fold more sensitive than the PCR assay for detecting *P. shigelloides* genomic DNA.

Reproducibility of the P. shigelloides hugA CPA assay. The intra-assay coefficient of variation (CV) was determined using various quantities of template DNA (20 ng, 200 and 2.0 pg) 10 times in a single run. The inter-assay CV was determined by performing the CPA assay using the same templates in 10 separate runs. The intra-assay CV ranged from 0.9 to 1.36%, and the inter-assay CV ranged from 0.99 to 1.71% (Table III). The reproducibility of the *P. shigelloides hugA* CPA assay was, therefore, good.

P. shigelloides hugA CPA efficacy in human stool specimens. The LoD of the *P. shigelloides hugA* CPA assay on human stools containing measured concentrations of *P. shigelloides* was examined. The CPA assay identified the presence of *P. shigelloides* in stools containing as little as $3x10^3$ CFU per g stool (Fig. 4A), whereas PCR had a LoD of $3x10^4$ CFU/g stool (Fig. 4B).

Utility of the P. shigelloides hugA CPA assay for detection in clinical and environmental samples. The *P. shigelloides* hugA CPA assay, PCR and culture-based detection were used to detect *P. shigelloides* in 100 clinical and environmental specimens (70 stool samples from patients with diarrhoea and 30 environmental water samples). The *P. shigelloides hugA* CPA assay and PCR method detected *P. shigelloides* in 11 (15.7%) and 8 (11.4%) stool specimens, respectively (Table IV). In the case of water samples, 4 (13.3%) and 3 (10.0%) water samples were *P. shigelloides* positive by CPA and PCR, respectively (Table IV). The samples that were positive by PCR were also positive by CPA. *P. shigelloides* strains were successfully isolated from all the CPA positive samples. The CPA detection accuracy was 100% compared with the traditional culture method. All samples were subjected to culture-based detection. The *P. shigelloides hugA* CPA assay, therefore, appears to be more sensitive for the detection of *P. shigelloides* in clinical and environmental samples than conventional PCR.

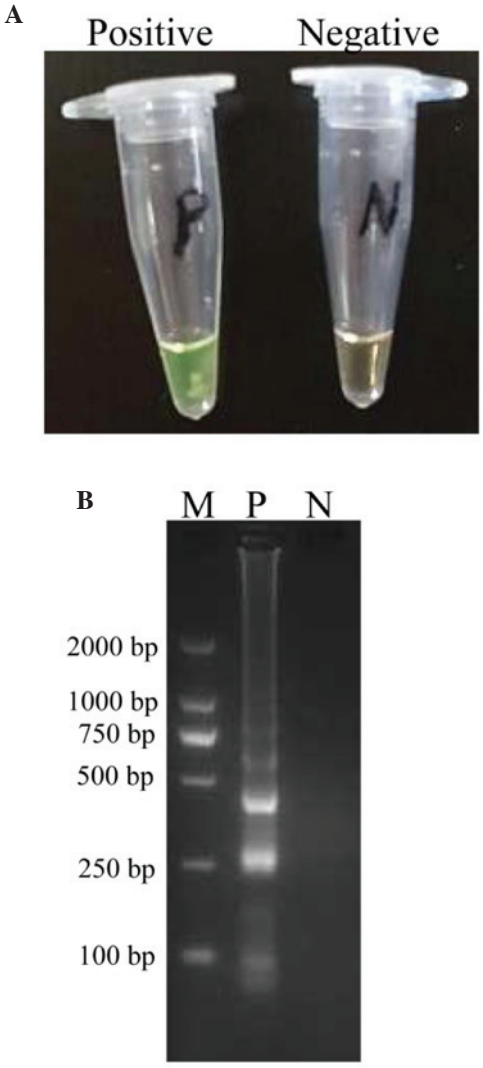

Figure 2. Confirmation and detection of the CPA products. (A) A colour change from orange to green in the presence of Loopamp Fluorescent Detection Agent indicated a positive CPA reaction, whereas retention of the orange colour of the reagent indicated a negative reaction. (B) Separation of positive and negative CPA reactions by electrophoresis on 2% agarose gels. CPA, cross-priming amplification; M, DL 2,000 bp DNA marke; P, positive CPA product; N, negative control.

Discussion

In the present study, a CPA assay was developed for the rapid detection of *P. shigelloides* as a potential on-site and

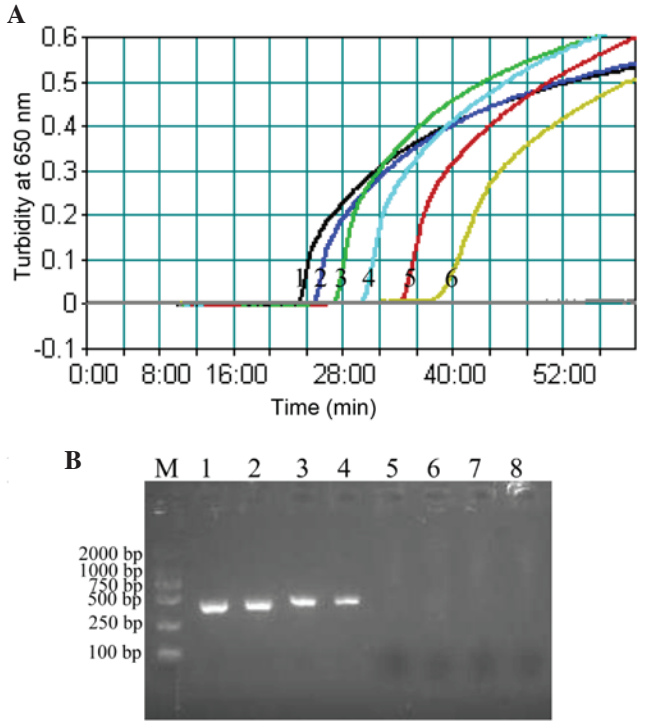

Figure 3. Sensitivity of the CPA and PCR methods. (A) Sensitivity of the CPA assay was assessed by measuring the turbidity (optical density at 650 nm) of reactions over the course of 60 min, using serial dilutions of *Plesiomonas shigelloides* ATCC 51903 genomic DNA as template (1, 20 ng; 2, 2 ng; 3, 200 pg; 4, 20 pg; 5, 2 pg; and 6, 200 fg per μl, respectively). A turbidity value of >0.1 within 60 min indicated a positive reaction. (B) Sensitivity of the PCR method was evaluated by detection of a 435-bp single target band by agarose gel electrophoresis, using serial dilutions of *Plesiomonas shigelloides* ATCC 51903 genomic DNA as template (1, 20 ng; 2, 2 ng; 3, 200 pg; 4, 20 pg; 5, 2 pg; 6, 200 fg; 7, 100 fg; and 8, 50 fg per μl). CPA, cross-priming amplification; PCR, polymerase chain reaction; M, DL 2,000 bp DNA marker.

point-of-care test in clinics. *P. shigelloides* is an important pathogen, which may contaminate food or aquatic environment and causes gastrointestinal illness (6-8). However, the current lack of a rapid and sensitive diagnostic method can result in inappropriate antimicrobial therapies being administered, potentially leading to further complications and fatal outcomes (12,28). Therefore, a rapid, sensitive, specific and economical detection method is urgently required.

The conventional methods for the isolation and identification of *P. shigelloides* involve enrichment in fluid media and subsequent isolation of colonies on selective media. Although extensively performed, the methods are labor-intensive and time-consuming, making it unsuitable for the rapid detection of causative pathogens associated with sporadic and outbreaks cases (25). As an alternative, various PCR-based assays have been developed for the detection of *P. shigelloides*. However, PCR-based methods require a high-precision thermal cycler, which restricts their widespread application and mean that these techniques are not suited to diagnosis of *P. shigelloides* in basic clinical and field laboratories in rural areas. Several isothermal amplification methods have been developed for the rapid diagnosis of infectious pathogens, including LAMP, which is a promising low-cost method for detecting various infectious pathogens (17-20). To date, the LAMP technique has been used to detect *P. shigelloides* in stool and environment specimens.

Table IV. Practical application of the *Plesiomonas shigelloides hugA* cross-priming amplification assay.

Detection method	Diarrhoea patient specimens (n=70)		Environmental water samples (n=30)	
	Positive	Negative	Positive	Negative
Polymerase chain reaction	8	62	3	27
Culture	11	59	4	26
Cross-priming amplification	11	59	4	26

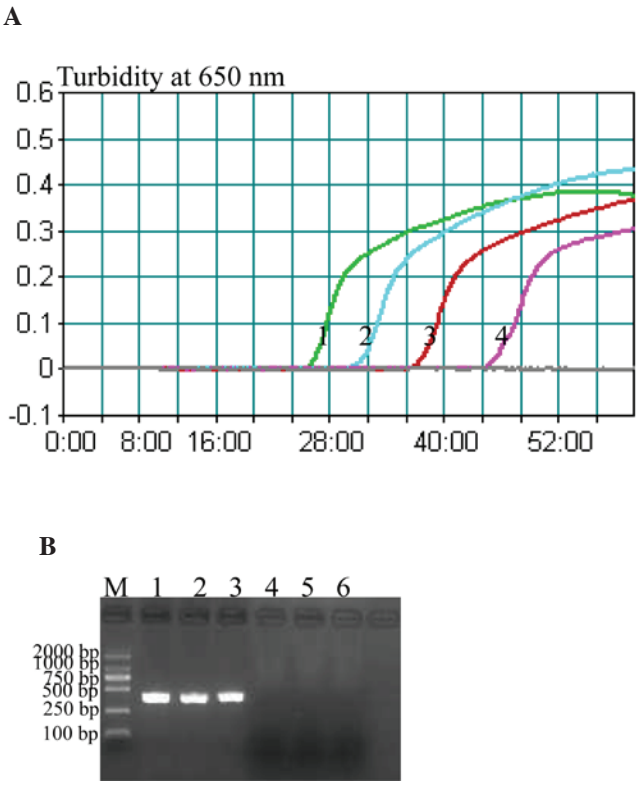

Figure 4. The detection limit of the CPA and PCR in spiked human stool. Stools from a healthy volunteer were spiked with (1) $3x10^6$, (2) $3x10^5$, (3) $3x10^4$, (4) $3x10^3$, (5) $3x10^2$ and (6) $3x10^1$ CFU *P. shigelloides* per g stool, prior to DNA extraction. (A) Detection by CPA and measurement of the turbidity (optical density at 650 nm) of reactions over the course of 60 min. A turbidity value of >0.1 within 60 min indicated a positive reaction. (B) Detection by PCR and agarose gel electrophoresis. CPA, cross-priming amplification; PCR, polymerase chain reaction; M, DL 2,000 bp DNA marker.

However, LAMP assays require primers with high stringency, for which primer design is complicated and requires specific software (Primer Explorer V4 softwarEiken Chemical Co. Ltd.), therefore, posing an obstacle for clinical application (16). Moreover, in LAMP, an additional step of DNA template denaturation is required (29). The CPA assay reported in the study does not require a denaturation step, does not require specific software for primer design, and as the gene target sequence used for primer design in the *P. shigelloides hugA* CPA assay is shorter than required for the LAMP assay, the subsequently reduced detection time is conducive to clinical application, as

described by Fang *et al* (30) for the detection of *M. tuberculosis* in sputum samples. CPA is a powerful innovative gene amplification technique, which has been described as an easy and rapid diagnostic tool for the detection of pathogens (27). The equipment requirements for the CPA assay are also limited to a heat block or water bath, maintaining a constant temperature of 63˚C for 1 h. The measurement of CPA products is possible by measuring turbidity, electrophoresis of amplicons or visual observation when using the Loopamp Fluorescent Detection Reagent. These features establish the CPA assay as a suitable method for *P. shigelloides* detection in basic clinical and field laboratories.

A 128-nucleotide fragment of the *hugA* gene was selected as the target for the CPA assay primers, as this gene is highly conserved in *P. shigelloides* strains (25). Primer specificity was determined by subjecting 33 non-*P. shigelloides* strains (causing similar clinical syndromes to *P. shigelloides*) to the *P. shigelloides hugA* CPA assay, revealing 100% specificity of the CPA assay for *P. shigelloides*. Positive amplification was completed by visual inspection, and no positive reactions were observed in the assays of non-*P. shigelloides* strains. The results of the present study suggested that the CPA assay for the detection of the gene that encodes the HugA outer membrane receptor required for heme iron utilization by *P. shigelloides* may be a reliable method to detect *P. shigelloides*. This procedure combined with an enrichment step allows *P. shigelloides* detection in clinical and environment specimens.

To the best of our knowledge, the present study is the first to use CPA technology to detect *P. shigelloides* in clinical and environmental specimens. The *P. shigelloides hugA* CPA method was 100-fold more sensitive than conventional PCR methods, detecting as little as 200 fg DNA per reaction. Several previous studies have also demonstrated that CPA has greater sensitivity than PCR for pathogen detection (17,29,31-34). Thus, the *P. shigelloides hugA* CPA assay is more appropriate than PCR for simple, rapid and sensitive detection of *P. shigelloides*.

To evaluate the practical application of the *P. shigelloides hugA* CPA assay for detection of *P. shigelloides* in clinical samples, 100 specimens of clinical and environmental origins were analysed using conventional culture-based detection detection, PCR, and the *P. shigelloides hugA* CPA assay. The *P. shigelloides hugA* CPA assay exhibited greater *P. shigelloides* detection capability than PCR, which was supported by several previous studies (17,29,31-34). The conventional PCR method also led to false negative results that were detected by the *P. shigelloides hugA* CPA assay; 3 stool specimens and 1 water sample were positive by culture and CPA, but PCR did not detect *P. shigelloides* in these samples. The reduced detection rate of PCR may be due to copy numbers of the *P. shigelloides* template that were less than the LoD, or the presence of PCR-specific inhibitors that may have affected the reaction sensitivity.

In conclusion, to the best of our knowledge, this is the first report of a CPA assay for the rapid detection of *P. shigelloides*. Compared with currently existing PCR methods, the *P. shigelloides hugA* CPA assay offers the advantages of improved sensitivity, rapidity, detection capability and ease of operation. In general, the CPA assay provides increased flexibility for clinical applications, and the isothermal amplification feature provides a potential method for the simple and rapid detection of *P. shigelloides* in basic clinical and field laboratories with limited resources.

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