Rapid and Sensitive Detection of *Plesiomonas shigelloides* by Loop-Mediated Isothermal Amplification of the *hugA* Gene

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**Abstract**

*Plesiomonas shigelloides* is one of the causative agents of human gastroenteritis, with increasing number of reports describing such infections in recent years. In this study, the *hugA* gene was chosen as the target to design loop-mediated isothermal amplification (LAMP) assays for the rapid, specific, and sensitive detection of *P. shigelloides*. The performance of the assay with reference plasmids and spiked human stools as samples was evaluated and compared with those of quantitative PCR (qPCR). No false-positive results were observed for the 32 non-*P. shigelloides* strains used to evaluate assay specificity. The limit of detection for *P. shigelloides* was approximately 20 copies per reaction in reference plasmids and 5 × 10^3 CFU per gram of spiked human stool, which were more sensitive than the results of qPCR. When applied in human stool samples spiked with 2 low levels of *P. shigelloides*, the LAMP assays achieved accurate detection after 6 h enrichment. In conclusion, the LAMP assay developed in this study is a valuable method for rapid, cost-effective, and simple detection of *P. shigelloides* in basic clinical and field laboratories in the rural areas of China.

**Introduction**

*Plesiomonas shigelloides* is a motile, oxidase-positive, facultatively anaerobic, gram-negative rod bacterium, which is presently classified in the family *Vibrionaceae* [1]. *P. shigelloides* has been isolated from a variety of environmental sources, primarily aquatic [2–4], and is distributed worldwide. Moreover, *P. shigelloides* has been associated with seafood-associated outbreaks [5]. *P. shigelloides* has been implicated as an agent of human gastroenteritis for many years, with an increasing number of reports describing such infections during the recent years [6]. This bacterium is also of considerable clinical importance as the etiological agent responsible for different types of opportunistic infections [7].

Although extra-intestinal infections such as septicemia, cellulitis, and meningitis caused by *P. shigelloides* are rarely reported, it has been associated with secondary infections in immunocompromised patients [8–10]. Salerno et al [12] also described an infection of *P. shigelloides* with a fatal outcome in a newborn. Since most laboratories concentrate on recovery of *Salmonella*, *Shigella*, *E. coli* and other classical enteropathogens, *P. shigelloides* may be overlooked during routine culture of stool samples. The lack of routine analysis for *P. shigelloides* in cases of gastroenteritis leads to only sporadic and occasional identification of this bacterium [11]. However, the greatest challenge to clinicians and epidemiologist is the lack of a rapid, early, and accurate diagnostic method for the detection of *P. shigelloides* as an emerging infectious disease in China.

Several methods such as culture studies and biochemical assays have been developed for detection and identification of *P. shigelloides*. Despite their effectiveness and accuracy, these assays are time consuming, usually requiring up to 5 days to complete. The isolation of *P. shigelloides* from clinical samples has often been unsuccessful owing to the fastidious nature of the organism and the low level of transient bacteremia associated with the disease process. Rapid, specific, and sensitive nucleic acid amplification tests (NAATs) such as standard and real-time PCR have been developed to detect *P. shigelloides* by targeting genes encoding for major virulence factors [5,13–14]. The major limitation to the widespread use of these assays is the fact that a sophisticated thermal cycler is an indispensable requirement of such tests, thereby limiting their wide applicability.

Recently, a novel NAAT technology termed loop-mediated isothermal amplification (LAMP) has attracted a great deal of attention as a rapid, accurate, and cost-effective method for detection of pathogens in clinical diagnostics [15,16]. LAMP employs 4–6 specially designed primers and a strand-displacing Bst DNA polymerase (isolated from *Bacillus stearothermophilus*) to amplify up to 10^14 target DNA copies under isothermal conditions (60°C–65°C) within an hour [15], making LAMP a potentially rapid and simple diagnostic tool for detection of *P. shigelloides* infection. In this study, we aimed to develop a rapid, sensitive, and highly specific LAMP assay to detect *P. shigelloides* and evaluate the assay performance with pathogen-simulated human stool.

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*P. shigelloides* is one of the causative agents of human gastroenteritis, with increasing number of reports describing such infections in recent years. In this study, the *hugA* gene was chosen as the target to design loop-mediated isothermal amplification (LAMP) assays for the rapid, specific, and sensitive detection of *P. shigelloides*. The performance of the assay with reference plasmids and spiked human stools as samples was evaluated and compared with those of quantitative PCR (qPCR). No false-positive results were observed for the 32 non-*P. shigelloides* strains used to evaluate assay specificity. The limit of detection for *P. shigelloides* was approximately 20 copies per reaction in reference plasmids and 5 × 10^3 CFU per gram of spiked human stool, which were more sensitive than the results of qPCR. When applied in human stool samples spiked with 2 low levels of *P. shigelloides*, the LAMP assays achieved accurate detection after 6 h enrichment. In conclusion, the LAMP assay developed in this study is a valuable method for rapid, cost-effective, and simple detection of *P. shigelloides* in basic clinical and field laboratories in the rural areas of China.
Materials and Methods

Ethics statement

Feces samples were acquired with the written informed consent from a healthy donor. This study was reviewed and approved by the ethics committee of the National Institute for Communicable Disease Control and Prevention, China CDC, according to the medical research regulations of the Ministry of Health, China.

Bacterial strains and culture conditions

A total of 32 strains (20 P. shigelloides and 20 non-P. shigelloides strains, as described in Table 1) was used for specificity testing. The bacterial load of the strains used for specificity evaluation was 10^5 pg/μL, which is high enough to avoid the false-negative amplification. Strain ATCC 51903 was used for the assay optimization, sensitivity evaluation, and simulating human stool samples. P. shigelloides and Enterobacteriaceae were cultured at 37°C overnight on brain heart infusion agar (BHI; BD Diagnostic Systems, Sparks, MD, USA). Non-Enterobacteriaceae strains were grown on blood agar, except for Vibrio strains, for which tryptose soy agar (TSA) supplemented with 2% NaCl was used. Campylobacter strains were grown under microaerophilic conditions (85% N2, 10% CO2, and 5% O2).

LAMP primers and reaction conditions

A set of 6 primers targeted toward the hugA gene of the species P. shigelloides were designed using PrimerExplorer V4 software (Eiken Chemical Co. Ltd., Tokyo, Japan) based on the conserved sequences determined by the alignment of the hugA gene sequences obtained from GenBank. The primers shown in Table 2 were synthesized by Sangon Biotech (Shanghai, China). The primer sequences and their positions in the expression site of the hugA gene are shown in Fig. 1. All LAMP reactions were performed with the Loopamp Kit (Eiken Chemical Co. Ltd., Tokyo, Japan) in a 25-μL mixture containing 1.6 μM FIP and BIP primers (each), 0.8 μM LF and LB primers (each), 0.2 μM F3 and B3 primers (each), 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 8 mM MgSO4, 100 μM (NH4)2SO4, 0.1% Tween 20, 0.8 M betaine, 1.4 mM deoxy nucleoside triphosphates (dNTPs) each, and 1 μL of Biol DNA polymerase (8 U/μL). The reaction mixture was incubated in a real-time turbidimeter LA320 (Teramecs, Tokyo, Japan) at 65°C for 60 min, followed by 80°C for 5 min to terminate the reaction. Positive and negative samples were distinguished from one another by a turbidity cutoff value of 0.1. After amplification, the LAMP products were detected by electrophoresis on 2% agarose gels with ethidium bromide staining or were determined by visual inspection after adding 1 μL of 1,000× SYBR green I.

Reference plasmid

To determine the sensitivity of the LAMP assay, a recombinant plasmid containing the target sequence of the hugA gene from the P. shigelloides strain (ATCC 51903) was constructed as follows: 1) A pair of primers was designed to span the sequences between the F3 and B3 primers; forward primer hugA-F (5′-GCGGTCTCCGGTTCGAAT-3′) and reverse primer hugA-R (5′-GTTACCGGGTCTGTTATG-3′); 2) the PCR products (259 bp) were cloned into the pEASY-T1 vector using the pEASY-T1 Cloning Kit (Transgen, Beijing, China); 3) the recombinant plasmid was quantified with a NanoPhotometer (Implen, Munich, Germany) and was serially diluted to concentrations of 1×10^6, 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 copies/μL in order to evaluate the limit of detection and the reproducibility of the LAMP assay.

Table 1. Strains used in this study.

| Latin name | Strain | Strains number |
|------------|--------|---------------|
| Plesiomonas shigelloides | ATCC51903 | 1 |
| | Isolated strains | 19 |
| Enteropathogenic E. coli | Isolated strain | 1 |
| Enterotoxigenic E. coli | Isolated strain | 1 |
| Enteroinvasive E. coli | Isolated strain | 1 |
| Enterohemorrhagic E. coli | EDL933 | 1 |
| Enteraggregative E. coli | Isolated strain | 1 |
| Salmonella enterica | ATCC14028 | 1 |
| Shigella flexneri | Isolated strain | 1 |
| Shigella sonnei | ATCC25931 | 1 |
| Salmonella typhi | H98125 | 1 |
| Klebsiella pneumoniae | ATCC700603 | 1 |
| Proteus vulgaris | Isolated strain | 1 |
| Aeromonas veronii | 1.2205 | 1 |
| Clostridium perfringens | Isolated strain | 1 |
| Enterobacter cloacae | Isolated strain | 1 |
| Serratia marcescens | Isolated strain | 1 |
| Vibrio para-haemolyticus | ATCC17802 | 1 |
| Staphylococcus aureus | ATCC6538 | 1 |
| Streptococcus pneumoniae | Isolated strain | 1 |
| Streptococcus pyogenes | Isolated strain | 1 |
| Streptococcus sanguis | Isolated strain | 1 |
| Streptococcus salivaricus | Isolated strain | 1 |
| Streptococcus bovis | Isolated strain | 1 |
| Enterococcus faecalis | ATCC35667 | 1 |
| Yersinia enterocolitica | ATCC23715 | 1 |
| Pseudomonas aeruginosa | ATCC15442 | 1 |
| Aeromonas hydrophila | ATCC7966 | 1 |
| Listeria monocytogenes | 54003 | 2 |
| Enterobacter sakazakii | ATCC51329 | 1 |
| Campylobacter jejuni | ATCC33291 | 1 |
| Vibrio minicis | Isolated strain | 1 |
| Vibrio vulnificus | Isolated strain | 1 |
| Vibrio fluvialis | Isolated strain | 1 |

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Evaluation of the sensitivity, specificity, and reproducibility of the LAMP assay

To compare the sensitivities of the LAMP assay and quantitative PCR (qPCR), the serially diluted reference plasmids (at concentrations of 1×10^6, 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 copies/μL containing the target DNA) were used to define the limit of detection. The qPCR assay was performed with the primers and probe in Table 2. qPCR amplification was performed in a 20-μL reaction volume containing 0.25 μM primer (each), 0.18 μM probe, 1× Premix (Takara Bio, Inc., Otsu, Japan) Ex TaqTM, and 2 μL of DNA template. The assays were conducted using the PCR settings of pre-denaturation at 95°C for 30 s, 40 cycles of denaturation at 94°C for 5 s, and extension at 60°C for 34 s in an ABI PRISM system (Applied Biosystems, Carlsbad, CA, US). Fluorescence readings were acquired using the 6-carboxyfluorescein (FAM) channel.
Genomic DNA of the 32 non-\textit{P. shigelloides} strains were detected by LAMP to determine the specificity of the \textit{hugA} LAMP assay. All detection assays were performed in triplicate.

A set of 3 reference plasmids with varying concentrations (10^6, 10^4, and 10^2 copies/μL) was amplified in two ways (10 times on 1 day and once on each of 10 days) to evaluate the reproducibility of the LAMP assay. The intra-assay coefficient of variation (CVi) and inter-assay coefficient of variation (CVo) were analyzed at the time of peak precipitation, as measured by turbidity on a real-time turbidimeter. Statistical analyses were conducted using SAS software version 9.1.

**Results**

**Specificity of the LAMP assay**

The specificity of the LAMP assay targeting \textit{hugA} gene was tested with 52 bacterial strains (Table 1). Positive amplifications were observed in 20 \textit{P. shigelloides} strains within a 60-min incubation period. By contrast, 32 non-\textit{P. shigelloides} strains were not amplified after a 60-min incubation period. This result indicates that no false-positive amplifications were observed with these heterologous species in the LAMP assay.

**Sensitivity of LAMP assay**

The limit of detection of LAMP (Fig. 2A) and qPCR for the \textit{hugA} gene were 20 and 200 copies/reaction, respectively. This result indicates that the LAMP assay is more sensitive than qPCR for detecting \textit{P. shigelloides} DNA. The LAMP products could also be detected by electrophoresis (Fig. 2C) and visual inspection after adding 1 μL of 1,000× SYBR green I (Fig. 2D).

**Table 2.** LAMP and qPCR primers used in this study to detect \textit{P. shigelloides}.

| Assay type | Primer/Probe name | Sequence (5'–3') | Position |
|------------|------------------|-----------------|----------|
| \textit{hugA}-LAMP | F3 | AACACGTTCGGACCCATC | 3776–3973 |
| | B3 | ACTTACGGCCGAGCAAGAAG | 3958–3977 |
| | FIP | CGTTACGGCAGGGTTCGTTAGTGACGATACGGTGGT | 3846–3867, 3806–3824 |
| | BIP | GTCAGCCAAAACTGCTGGATGGATGGCAG | 3878–3897, 3940–3957 |
| | LF | ACCGACGCTGAAGAGATGGT | 3825–3844 |
| | LB | GCGACAGGTGATCTTCGAT | 3918–3938 |
| \textit{hugA}-qPCR | F | GGATATTCGCCGCTTACAT | 4022–4040 |
| | R | TATGCCGGCATATTATTA | 4121–4137 |
| | Probe | FAM-CCTCATGGTGCTGCCCGGCAAT-BHQ-1 | 4046–4070 |

Figure 1. Names and locations of target sequences used as primers for the expression site of \textit{hugA} LAMP. doi:10.1371/journal.pone.0041978.g001
Reproducibility of LAMP assay

The CVi was assessed by testing 3 reference plasmids with varying concentrations (10^6, 10^4, and 10^2 copies/µL), 10 times in a single run, whereas the CVo was assessed by testing the same plasmids 10 times in 10 separate runs. The CVi ranged from 1.21% to 1.54%, while the CVo ranged from 2.17% to 3.23%.

Evaluation of LAMP assay in simulated human stool

The detection limit of LAMP in simulated human stool was also examined. The LAMP assays detected the presence of P. shigelloides strains down to as little as 5×10^3 CFU/g. By comparison, the qPCR assays had a detection limit of 5×10^4 CFU/g for hugA gene in simulated human stool samples (data not shown). Table 3

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**Figure 2. Real-time sensitivity and detection limit of hugA-LAMP.** (A) Real-time sensitivity of hugA-LAMP as monitored by the measurement of turbidity (optimal density at 650 nm). A turbidity of >0.1 was considered to be positive for hugA-LAMP. The detection limit was 20 copies/reaction. (B) The relation between the threshold time (Tt) of each sample and the log copies/reaction. The standard curve was drawn on the basis of 3 independent repeats and the linear relationship R^2 = 0.9787. (C) Sensitivities of electrophoretic analysis of hugA-LAMP amplified products. Lane M: DL2000 marker; lane 1: 2×10^6 copies/reaction; lane 2: 2×10^5 copies/reaction; lane 3: 2×10^4 copies/reaction; lane 4: 2×10^3 copies/reaction; lane 5: 2×10^2 copies/reaction; lane 6: 2×10^1 copies/reaction; lane 7: 2×10^0 copies/reaction; lane 8: no template. (D) SYBR green I fluorescent dye-mediated monitoring of hugA-LAMP assay amplification. The original orange color of the SYBR Green I changed to green in case of positive amplification, whereas the original orange color was retained for a negative control with no amplification.

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summarizes LAMP and qPCR results in human stool samples spiked with low levels of P. shigelloides strains after various enrichment periods (4, 6, 8, 10, 12, and 24 h). In this graph, the human stool sample was spiked with 1.3 CFU of Strain ATCC 51903. We observed that the LAMP assay performed well for the detection of P. shigelloides in basic clinical and field laboratories in rural areas.

Although the pathogenesis of P. shigelloides-associated gastroenteritis has not yet been elucidated, a number of potential virulence factors have been described [19,20]. Acquisition of iron is known to be involved in the virulence of a variety of bacterial pathogens [21,22]. Heme is the most abundant source of iron in the body, and many pathogenic bacteria possess heme transport systems. The hugA gene, one of the characterized genes encoded in the heme iron utilization system of P. shigelloides, encodes an outer membrane receptor that is required for heme iron utilization.

In this study, all hugA gene sequences of P. shigelloides recorded in the GeneBank were aligned, and the LAMP primers were designed on the basis of the conserved regions. We tested 32 non-P. shigelloides strains to evaluate the specificity of the hugA LAMP assay for the bacteria, with the results showing that the specificity of the LAMP assay was 100%.

To the best of our knowledge, this is the first study applying the novel LAMP technology for the detection of P. shigelloides in human stool. Previously, spiked samples were usually enriched overnight without characterizing the effects of different enrichment times on the detection outcomes [23,24]. In this study, P. shigelloides strain ATCC 51903 was used in experiments with simulated human stool samples, with the LAMP assays having a detection limit of 5×10^2 CFU/g stool. Positive detection occurred after a 6-h period of enrichment, and consistently thereafter, for the human stool samples spiked with 2 low levels [1 to 2 and 10 to 20 CFU/0.5 g] of ATCC 51903. We observed that the LAMP assay performed better than qPCR with respect to detection limit and assay speed in spiked human stool. In general, molecular level-based detection methods such as PCR and LAMP are subjected to a variety of inhibitors present in clinical samples. Some researchers have reported that the Bst polymerase in LAMP is less sensitive to the presence of inhibitors than the Taq polymerase used in classic PCR [25,26]. Our results showed that the LAMP assay is more accurate and sensitive than qPCR methods using simulated human stool samples, and proved markedly faster than qPCR by at least 20 min, thereby significantly shortening the total assay time.

In conclusion, the LAMP assay was successfully validated in this study for rapidity, sensitivity, specificity, and robustness; thus, this assay may serve as an effective means for screening P. shigelloides in

Table 3. Comparison of LAMP and qPCR assays in human stool samples spiked with low levels of P. shigelloides.

| Cell level (no. of CFU/0.5 g) | LAMP Tt (min) after enrichment | qPCR CT (cycles) after enrichment |
|-------------------------------|--------------------------------|---------------------------------|
|                               | 4 h   | 6 h   | 8 h   | 10 h  | 12 h  | 24 h  | 4 h   | 6 h   | 8 h   | 10 h  | 12 h  | 24 h  |
| 1–2                           | Not available | 30.3 | 27.1 | 25.2 | 22.7 | 22.4 | Not available | 36.5 | 33.2 | 30.8 | 29.6 | 29.1 |
| 10–20                         | Not available | 28.5 | 25.3 | 23.8 | 21.5 | 21.3 | Not available | 33.7 | 29.1 | 27.7 | 26.3 | 25.8 |

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Figure 3. A typical LAMP amplification graph generated when testing human stool samples spiked with the low level of P. shigelloides strain after various enrichment periods (4, 6, 8, 10, 12, and 24 h). In this graph, the human stool sample was spiked with 1.3 CFU of Strain ATCC 51903.
clinical samples. We proved that the LAMP assay demonstrated superior performance to qPCR in simulated human stool samples, and may facilitate rapid and reliable diagnosis of *P. shigelloides* infections in basic clinical and field laboratories in rural areas.

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**Author Contributions**

Conceived and designed the experiments: SM CY JX. Performed the experiments: SM. Analyzed the data: SM YX. Contributed reagents/materials/analysis tools: SM YX. Wrote the paper: SM CY.