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ORIGINAL ARTICLE

Development of a loop-mediated isothermal amplification assay for rapid detection of *Yersinia enterocolitica* via targeting a conserved locus

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ABSTRACT

Background and Objectives: Loop-mediated isothermal amplification is a novel nucleic acid amplification assay providing as a simple diagnostic tool for rapid identification of microbial diseases in developing countries. In this study, a LAMP assay was established for *Yersinia enterocolitica*, a leading cause of acute enterocolitis in young children.

Materials and Methods: LAMP assay was established with four primers targeting a specific locus for the detection of *Y. enterocolitica*. The assay was conducted at 65°C in thermo block for 90min. The sensitivity of LAMP was evaluated in comparison to conventional PCR using pTZ57R containing the target locus. Finally, specificity was assessed using DNA from common enteropathogenic bacteria.

Results: Results showed that the sensitivity of LAMP assay was 44-copy number, which was 10-fold higher than that of PCR. No cross-reactivity was observed when testing against other enteropathogenic pathogens.

Conclusion: This study showed that LAMP assay is an alternative molecular diagnostic tool for infections with *Y. enterocolitica*. In addition, this method may be useful in diagnosis at field or in laboratories without PCR machine.

Keywords: *Yersinia enterocolitica;* Loop-mediated isothermal amplification (LAMP), specific locus

INTRODUCTION

Yersinia enterocolitica is an important gastrointestinal pathogen belonging to enterobacteriaceae family. The bacterium has six biogroups and several serotypes, some of which are related with yersiniosis in humans and animals (1). The serotypes of O3, O8 and O9 have been frequently reported from many countries in the world. Yersiniosis due to *Y. enterocolitica* presents with acute enterocolitis with main symptoms of bloody diarrhea, abdominal pain, vomiting, fever and headache (2). The bacterium is transmitted via fecal-oral route most often by consumption of contaminated food or water (3). Immunological manifestations have also been reported in addition to abdominal symptoms. Reactive arthritis due to this organism is well recognized in Northern Europe, and presents with fever and swollen joints (4). Routine methods for identification of *Y. enterocolitica* include culture, serology and molecular assays (5, 6). The culture has been considered as gold standard method-for isolation of bacterium from stool specimens, for which Cefsulodin-Irgasan-Novobiocin agar (CIN) is the selective medium. The use of enrichment techniques such as cold enrichment and alkali treatment of specimens has resulted in improved recovery of this bacterium (7). However, these techniques are time consuming and not cost-effective. In contrast to conventional techniques, molecular assays are dependent upon amplification of DNA or RNA and do not require high microbial load or presence of

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active viable organisms (8). Moreover, the molecular assays can be used for several organisms that cannot be grown or grow very slowly in culture (9). Advances in molecular assays have led to introduction of new molecular assays with high specificity and sensitivity. Loop-Mediated Isothermal Amplification (LAMP) assay is a technique that amplifies DNA under isothermal conditions. In this assay, a set of four specific primers are used that anneal to six distinct regions within the target sequence. The use of four primers (F3, B3, FIP and BIP) results in high specificity and efficiency (10). Adding the loop primers enhances the performance and rapidity of LAMP assay (11). Using the loop primers also can reduce the amplification time to less than 30 minutes. After a short amplification time, a white precipitate is produced due to production of magnesium pyrophosphate as a by-product. Therefore, it can be observed by naked eye without any special processing or electrophoresis (12). Moreover, LAMP assay is not relatively affected by inhibitors remaining in DNA extraction step. Thus, relatively simple DNA extraction assays (e.g. boiling) can be used for DNA extraction instead of commercial DNA extraction kits (13). These features have resulted in the use of LAMP assay in identification of several pathogenic bacterial infections (14-17).

The objective of this study was development a Loop-Mediated Isothermal Amplification (LAMP) assay for detection of *Y. enterocolitica* based on a specific locus.

MATERIALS AND METHODS

Primer design. In this study, to identify a conserved locus, a whole genome BLAST analysis was conducted against *Yersinia enterocolitica* complete genomes (Accession No: CP002246.1, FR729477.2, AM286415.1, HF571988.1 and CP007448.1). The BLAST results revealed a specific sequence encoding a hypothetical protein (Table1). This locus was selected for primer design. Four primers were designed using PrimerexplorerV4 (https://primerexplorer.jp/lamp4) based on software default settings. All of the primer sequences are shown in Table 2.

Table 1. *Yersinia enterocolitica* complete genomes and positition of the considered locus

Ref seq complete genomes	Position on whole genome
Yersinia enterocolitica W22703 biovar 2	42803-42023
Yersinia enterocolitica subsp. palearctica 105.5R	1679158-1678378
Yersinia enterocolitica subsp. palearctica Y11	1770666-1771446
Yersinia enterocolitica subsp. enterocolitica 8081	3107444-3108224
Yersinia enterocolitica (type O:5) YE53/03	3254299-3255079
Yersinia enterocolitica LC20	1646941-1646162

Table 2. Primers used in LAMP assay

Primer	Sequence(5'->3')
F3	CCATCTGCTGCTAATTGGGT
B3	AAAACTGGACGGTGCAAGA
FIP	GGCGGGTATCGCCTGGTATTTGAAATTCAGCAACTCAGCCATCTC
BIP	TCTGCGCCGCAGCTTCTTTTAAAATTCGGCACTGGATGACAG

Bacterial strains and DNA extraction. A standard (ATCC 23715) and six clinical strains of *Y. enterocolitica* were cultured on LB broth at 37°C for 24 hours. DNA extraction was conducted using DNA extraction kit (Promega Wizard Genomic DNA purification kit, Madison, WII, USA) according to the manufacturer's instructions. Purity of extracted DNA was determined based on measurement of OD260 nm/280 nm using Nano Drop instrument (Thermo Scientific), and calculated to be 1.8.

Optimization of LAMP assay. LAMP reaction mixture was prepared with a total volume of 25 µl, containing 2.5 µL of 10X ThermoPol Buffer (New England Biolabs, Massachusetts, USA), 0.2 pmol each of F3 and B3 primers, 0.8 pmol each of FIP and BIP primers, different concentrations of dNTPs (0.4 mM, 0.6 mM, 0.8 mM; 1 mM and 1.2 mM), different concentrations of MgSO4 (3 mM, 4 mM, 5 mM and 6 mM), 8 U Bst DNA polymerase large fragment (New England Biolabs, Massachusetts, USA) and different concentrations of DNA template. The reaction mixture was incubated in a Thermoblock at several temperatures for different minutes, and finally the products were electrophoresed on 1% agarose gel.

Specificity of LAMP primers. The specificity of LAMP assay was evaluated using enteric pathogens, including *Shigella sonnei*, *Salmonella enteritidis*, *Escherichia coli O157H7, Aeromonas hydrophila*, *Vibrio cholera* and *Campylobacter jejuni.* The LAMP product was analyzed by gel electrophoresis.

Determination of sensitivity of LAMP assay

Cloning of the considered locus in pTZ57R vector: TA cloning procedure was done using CloneJET™ PCR cloning kit (Thermo Scientific) according to the manufacturer's instructions. The ligation reaction mixture was prepared at a PCR product/pTZ57R ratio of 5:1, and was incubated overnight at 8°C. The ligated product was transformed into competent *E. coli* DH5α cells, plated on LB agar plates with ampicillin (100 µg/ml) and incubated overnight at 37°C. Positive colonies were screened with colony PCR using F3 and B3 primers, and the recombinant plasmid was extracted from a single positive colony. The constructed plasmid was used to determine the detection limit of LAMP assay. For this reason, DNA concentration was measured using NanoDrop (Thermo Scientific), and gene copy number was calculated using the following formula (18):

(Mass [in grams] × Avogadro's number)/ (average molecular weight of base × template length) = molecules (gene copies) of DNA.

A 10-fold serial dilution of the constructed pTZ57R was prepared in sterile water, and LAMP assay and PCR (using F3 and B3 primers) were carried out using different DNA concentrations.

RESULTS

Polymerase chain reaction using outer primers. The PCR using outer primers (F3 & B3) produced the expected size of amplicon in all *Y. enterocolitica* strains tested. Fig. 1 shows the specific 232bp band obtained from standard and clinical strains.

Optimization of LAMP assay. To optimize and find an optimal incubation temperature for LAMP assay, a standard reaction was conducted with four temperatures (60-66°C) for 90 min. Maximum amplification was obtained at 64°C. Therefore, the optimal temperature for LAMP assay was established at 64°C and applied for all the subsequent applications. To optimize a best time for LAMP reaction amplification to yield enough observable products, four different times were applied for LAMP reaction, and good results were obtained at 90 min. Different $MgSO_4$ concentrations, which is important factor in LAMP reaction, were tested and the best result was obtained at 6 mM. As expected, the LAMP reaction failed in the absence of $MgSO_4$. Different concentrations of dNTPs were also tested in LAMP reactions, and maximum amplification was established at 1mM.

Specificity of LAMP and PCR assay. The specificity of PCR and LAMP assays was tested using DNA samples from *Shigella sonnei*, *Salmonella enteritidis*, *Escherichia coli O157H7*, *Aeromonas hydrophila, V.cholera* and *Campylobacter jejuni*. As shown in Fig. 2, no non-specific amplifications were observed when using DNA from the above-mentioned species.

Detection limit of assay. In order to determine the lower detection limit of LAMP and PCR (using F3and B3 primers), a 10-fold serial dilution (ranging from 10^{-1} to 10^{-8} of constructed plasmid) was provided. The results of amplification (Fig.3) showed that minimum

copy number of DNA detectable by LAMP and PCR assays was 44 and 440, respectively.

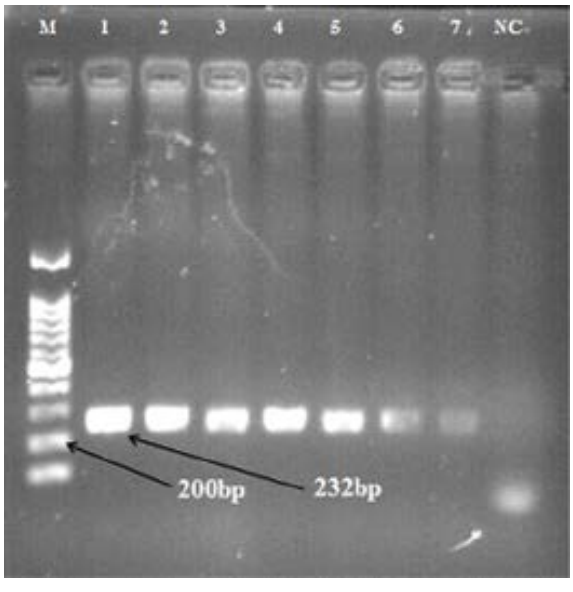

Fig. 1. Detection of *Y.enterocolitica* strains using LAMP outer primers. Lane M, 1500bp DNA ladder (Sinaclon, Tehran, Iran); Lane 1, *Y.enterocolitica* ATCC23715; Lane 2-7, *Y.enterocolitica* clinical isolates; NC, negative control.

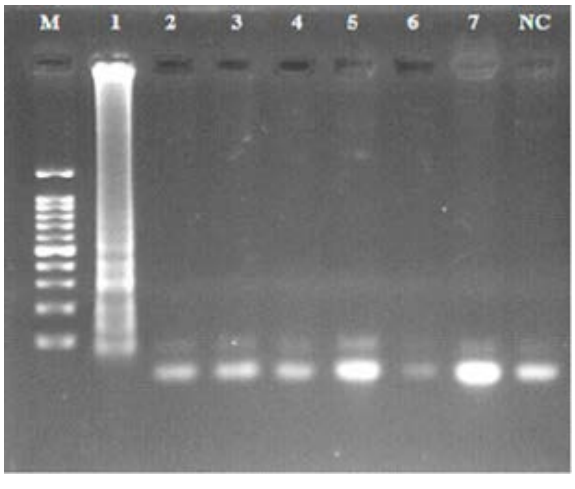

Fig. 2. Specificity of the LAMP assay. LAMP amplification products were electrophoresed on %2 agaros gel. Lane M, 1500bp DNA ladder (Sinaclon, Tehran, Iran); Lane 1, *Y.enterocolitica*; lane 2-7, *Shigella sonnei, V.cholera*; *Salmonella enteritidis, Escherichia coli o157H7, Aeromonas hydrophila* and *Campylobacter jejuni,* respectively; lane NC, negative control.

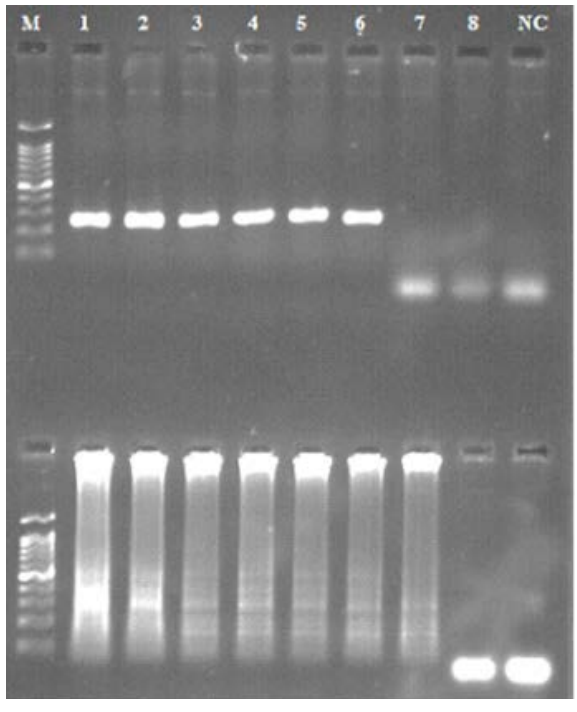

Fig. 3. Sensitivity of PCR (above) and LAMP (below) assays. Agarose gel electrophoresis of LAMP products from 10-fold serially diluted construct plasmid PTZ57R. Lane M, 1500bp DNA ladder (Sinaclon, Tehran, Iran); lane 1, dilution of 10^{-1}; lane 2, dilution of 10^{-2}; lane3, dilution of 10^{-3}; lane 4, dilution of 10^{-4}; lane 5, dilution of 10^{-5}; lane 6, dilution of 10^{-6}; lane 7, dilution of 10^{-7}; lane 8, dilution of 10^{-8}; NC, negative control.

DISCUSSION

In the present study, we successfully designed a LAMP assay targeting a conserved locus of *Y.enterocolitica*. This bacterium has many genes involved in pathogenicity, but there are limited studies targeting the virulence genes in molecular diagnosis of *Y.enterocolitica* (19; 20). However, in several researches, other genes such as *out*L, *ail*, *pho*P and *gyr*B were used for primer design (21-23). In the present study, we found an appropriate target using whole genome blast analysis. The results showed that the FR729477 locus has 1311bp and is conserved in all *Y.enterocolitica* complete genomes submitted in GenBank; therefore, the designed primers had 100% similarity to all representative *Y.enterocolitica* strains but no other species of *Yersinia*. In addition, the absence of a similar sequence in other relative species enhances

the specificity of primer. To our knowledge, there are no published reports on the use of the FR729477 locus for molecular detection of *Y.enterocolitica*.

We applied a new modification in the beginning of procedure according to strand-displacement activity of Bst polymerase to improve it. In this way, before adding Bst polymerase to LAMP mixture, a pre-heating temperature (80°C) was carried out and immediately cooled down over an ice bath. The results revealed that this step considerably increases the reaction rate.

The LAMP assay targeting the FR729477 locus was 100-fold more sensitive than conventional PCR, and was in the similar ranges as in a forementioned reports. The detection limit of LAMP assay was determined to be 44 copy number, which was 10-fold more sensitive than the conventional PCR using B3&F3 primers. Xu et al. described a LAMP assay for detection of *Y. enterocolitica* and compared it to conventional PCR. Their results showed that the LAMP assay is capable to detect 97 fg of genomic DNA (equivalent to 37 genome copies) of *Y. enterocolitica,* which is 100-fold more sensitive than PCR (24). Gao et al. developed a LAMP assay for the detection of *Y. enterocolitica* bioserotypes with primers targeting the *gyr*B. The latter LAMP assay was able to identify four different bioserotypes of *Y. enterocolitica* with a sensitivity about 31.6 fg DNA/reaction and specificity of 65 CFU/mL (25).

The amplification time of LAMP commonly varies from 60-120 min (26). The rate of amplification also accelerates using loop primers (LB&LF), which has been reported in the previous studies. However, in our study, the amplification period was 90 minutes without using LF and LB primers, indicating no noticeable difference in rate of reaction.

There are contradictory reports about the effect of betaine on LAMP amplification (27). A study by Njiru showed that the efficacy of LAMP amplification was enhanced using betaine with a concentration of 0.8 M (28). However, some reports state that betaine has no useful effect on LAMP amplification (29), and consequently Zhou et al. showed that betaine was not necessary when amplifying non-GC-rich target sequences (30). Due to reduction of base stacking, betaine reduces the formation of secondary structure in GC-rich regions (31).

When LAMP assay is correctly proceeded, an opalescent color changing is created, which can be visualized by the naked eye as turbidity. However, some modifications have been applied to accelerate the volatilization of LAMP reaction. Recently, Zhou et al. reported a simple visual detection method in which calcein and Mn^{2+} were used before the reaction (30). Moreover, using SYBER Green dye before adding LAMP reaction mixtures has been common in many studies (32; 33). In contrast to the above studies, in present research, only the opacity of reaction was considered.

In conclusion, this established LAMP assay is a quick and cost-effective approach and it can be used for diagnostic purposes in laboratories with limited equipment.

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