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Development and evaluation of probe based real time loop mediated isothermal amplification for *Salmonella*: A new tool for DNA quantification

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ABSTRACT

A one step, single tube, accelerated probe based real time loop mediated isothermal amplification (RT LAMP) assay was developed for detecting the invasion gene (InvA) of *Salmonella*. The probe based RT LAMP is a novel method of gene amplification that amplifies nucleic acid with high specificity and rapidity under isothermal conditions with a set of six primers. The whole procedure is very simple and rapid, and amplification can be obtained in 20 min. Detection of gene amplification was accomplished by amplification curve, turbidity and addition of DNA binding dye at the end of the reaction results in colour difference and can be visualized under normal day light and in UV. The sensitivity of developed assay was found 10 fold higher than taqman based qPCR. The specificity of the RT LAMP assay was validated by the absence of any cross reaction with other members of enterobacteriaceae family and other gram negative bacteria. These results indicate that the probe based RT LAMP assay is extremely rapid, cost effective, highly specific and sensitivity and has potential usefulness for rapid *Salmonella* surveillance.

1. Introduction

Isothermal amplification technologies such as nucleic acid sequence-based amplification (NASBA), transcription mediated amplification (TMA), strand displacement amplification (SDA) and rolling circle amplification (RCA) had been developed to avoid temperature cycling device needed in PCR-based methods. Notomi et al. (2000) developed loop-mediated isothermal amplification (LAMP) which makes use of the strand displacement activity of some DNA polymerases and the hairpin-forming tendency of primers. Chance of contaminations and false positive results are cumbersome factor of LAMP. In addition to this, LAMP cannot determine the number of DNA copies present in the samples. RT LAMP is a powerful gene amplification technique which is emerging as a specific, fast diagnostic tool for early detection and identification of microbial diseases. Real time LAMP can be done in a portable instrument in which the generation of fluorophores can be followed in real time to monitor the amplification of DNA like qPCR. It is having an advantage of an objective determination of fluorescence above a defined threshold as a comparison to visual assessment of turbidity or fluorescence. The mechanism of RT LAMP is similar to cascade rolling circle amplification, and is based on the principle of auto cycling strand displacement DNA synthesis and emittance of fluorophore from the probe (Fig. 1). Strand displacement by the inclusion of *Bst* DNA polymerase in the LAMP means that there is no need for the high temperatures normally required in template PCR amplification (Notomi et al., 2000). RT LAMP initiates at the F2c sequence of the target, with the Fd probe quenched through annealing to Q-FIP. This new strand is displaced by upstream synthesis from the F3 primer. The BIP primer annealed to the B2c sequence of newly synthesized strand displaces the Fd probe. This releases the quenching resulting in a gain of signal. The newly synthesized strand is displaced by extension from the B3 primer. The resulting structure undergoes exponential amplification in the RT LAMP reaction. Subsequent initiations at FIP give rise to additional release of Fd, resulting in exponential signal detection (Tanner et al., 2012). (See Fig. 2.)

2. Materials and methods

2.1. Bacterial samples

Twenty seven different strains including 12 *Salmonella* and 15 non-*Salmonella* strains listed in Table 1 were used in present study. *Salmonella* Typhimurium was used as reference strain. All *Salmonella* strains included in the study were procured from the repository of the National *Salmonella* Centre IVRI. Non-*Salmonella* strains were procured from Division of Veterinary Public Health, Division of Bacteriology and Mycology and General Bacteriology Lab, CADRAD, IVRI. All the isolates were tested for their purity, morphological and biochemical characteristics.

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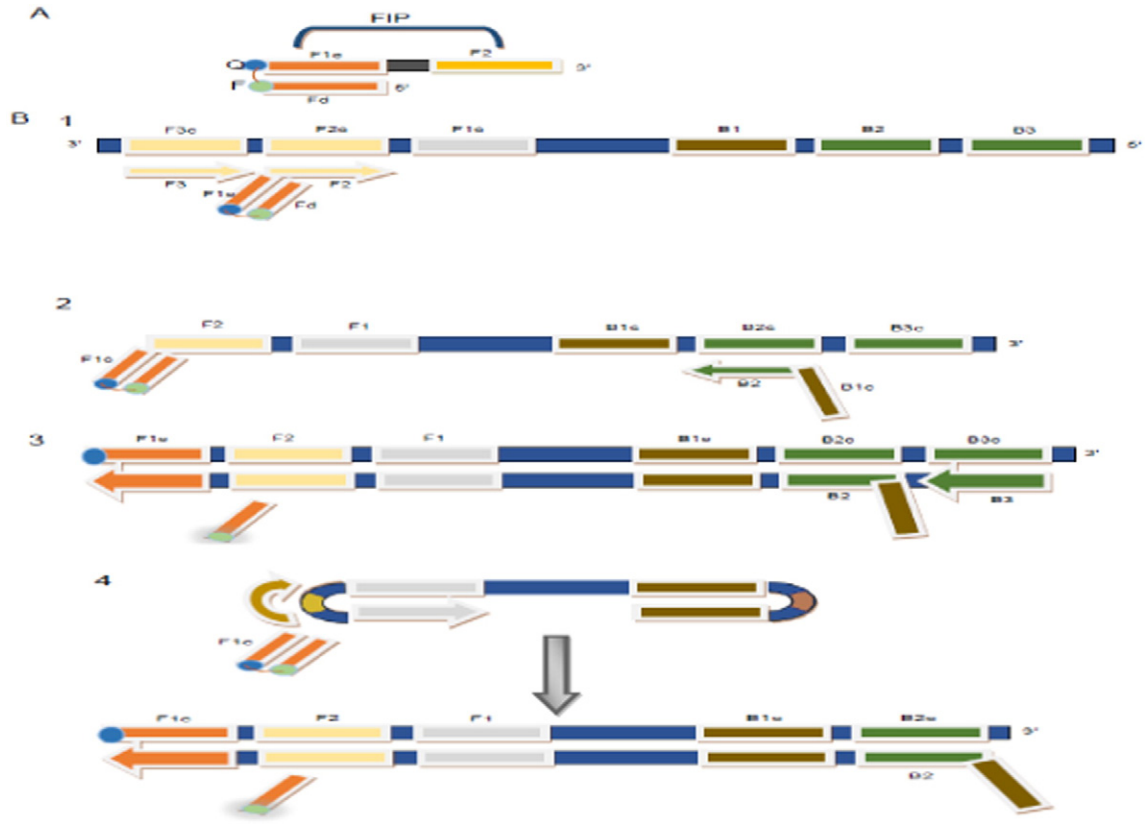

Fig. 1. CyIB in LAMP. (A) Schematic depiction of a CYIB probe, with a 5′-quencher FIP annealed to a 3′-fluorophore Fd. The quencher and fluorophore moieties are represented by Q and F, respectively. (B) Outline of CYIB LAMP reactions, with core LAMP primers FIP (F1c + F2), BIP (B1c + B2), F3 and B3, and the CYIB oligonucleotide, Fd (Q, blue; F, green). For clarity, LoopF and LoopB primers are not shown.

2.2. Nucleic acid extraction

DNA extracted from faeces using CTAB method according to Sambrook and Russel (1989) with certain modifications. Briefly, 2 ml of inoculated buffer peptone water was taken in a 2 ml microfuge tube. Then the sample was centrifuged at 12,000 rpm for 10 min, supernatant was decanted and pellet was washed with PBS. Subsequently 0.5 ml of TE buffer, 100 μl of 10X SDS and 5 μl of proteinase K (20 mg/ml) were added to the pellet. The tube was incubated for 1 h at 37 °C followed by addition of 100 μl of 5 M NaCl, 50 μl of 10% CTAB in 0.7 M NaCl and it was incubated again at 60 °C for 10 min. Equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added and centrifuged at 12,000 rpm for 10 min. Supernatant containing the aqueous phase was transferred to a new microfuge tube. Again equal volumes of chilled 100% ethanol, 0.1 volume of 3 M sodium acetate (pH 5.2) were added and the tube was spinned at 12,000 rpm for 10 min. Pellet was washed with 70% ethanol. The pellet was air dried and resuspended in 50 μl elution buffer. Two microlitres of Rnase A was added and incubated at 37 °C for 30 min. Finally extracted DNA was stored in −20 °C till further use. The DNA concentration was estimated by Nanodrop®.

2.3. Design of primers for real time LAMP

LAMP primers and probes were designed for *Salmonella* invasion gene (InvA gene; GenBank Accession No. U43273) using LAMP designer premier Biosoft International software. All the generated primers and probes were checked for their specificity by Basic Local Alignment Tool (BLAST) available in National Centre for Biotechnology Information (NCBI) website. 3′ of Fd was attached with fluorophore Cy5 and 5′ of FIP was attached with quencher Iowa black as shown in Table 2.

2.4. Optimization of RT LAMP

The RT LAMP was carried out in a total volume of 25 μl of reaction mixture. The initial standardization and optimization of the RT LAMP were carried out by using a set of four or six primers to compare the rapidity of amplification reaction. Briefly, the test was carried out at different temperatures (63 °C, 65 °C, 67 °C). LAMP reaction mixture was optimized in Real time thermocycler Mx3000P QPCR system (Agilent Technologies, Inc.) by using different concentrations of inner primers, probe (Integrated DNA Technologies Coralville, IA, USA), outer primers, loop primers, $MgSO_4$, dNTPs and Betaine (Sigma-Aldrich). In all reaction mixtures, 8 units of *Bsm* DNA polymerase (New England Biolabs) and 2 μl of DNA template were used. The RT LAMP products were interpreted by amplification curve with respect to time (Ct value). One Ct is equal to 1 min. DNA binding dye SYBR green I dye (1000×) around 1 μl was added at the end of the reaction into the tube which gave colour difference to positive and negative products which can be visualized under normal day light and in UV. The turbidity was also noticed in the reaction tubes due to formation of magnesium pyrophosphate, which had direct correlation with amplification.

2.5. qPCR

For confirming the presence of template DNA solutions, the serials dilutions of the template DNA were analysed by qPCR method using Mx3000P qPCR system (Agilent Technologies, Inc.). qPCR was performed by using mericon *Salmonella* RT PCR kit™ (Qiagen, Germany), as per the manufacturer's instruction. Briefly, amplification was carried out in the final volume of 20 μl reaction containing 10 μl of reconstituted mericon assay mixture in each tube and 10 μl of sample DNA template,