DETECTION OF *SALMONELLA ENTERICA* BY LOOP-MEDIATED ISOTHERMAL AMPLIFICATION OF DNA USING A FLUORESCENTLY LABELED LOOP PRIMER

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Salmonellosis remains one of the leading causes of bacterial gastrointestinal infections in humans and animals. Molecular diagnostics has dramatically reshaped the diagnostic landscape for Salmonella infection; however, it remains time- and resource-intensive. Isothermal DNA amplification, for example loop isothermal amplification (LAMP), performed at a constant temperature, is the basis for the development of rapid diagnostic tests that can be adapted to the point-of-care (PoC) formats and implemented in resource-limited settings or remote from centralized laboratories. The aim of this study was to develop and validate a novel LAMP-based method for detecting *Salmonella enterica* in human stool samples, wherein amplification results are monitored using a loop primer labeled with a fluorophore and an internal quencher. The proposed method achieves a limit of detection (LoD_{95}) of 250 copies per reaction, with a sensitivity of 86.84% (95% CI: 71.91–95.59%) and specificity of 96.49% (95% CI: 87.89–99.57%) relative to qPCR, and demonstrates increased robustness against DNA amplification inhibitors present in fecal samples. Incorporation of distinct fluorophores into loop primers for FLP-LAMP targeting different genes could potentially enable multiplexing and simultaneous detection of multiple pathogens, thereby expanding the diagnostic utility of isothermal amplification.

Keywords: *Salmonella enterica*, LAMP, fluorescent loop primer, fluorescent probe, analytical validation, DNA amplification inhibitors

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ДЕТЕКЦИЯ *SALMONELLA ENTERICA* ПЕТЛЕВОЙ ИЗОТЕРМАЛЬНОЙ АМПЛИФИКАЦИЕЙ ДНК С ИСПОЛЬЗОВАНИЕМ ФЛУОРЕСЦЕНТНО МЕЧЕНОГО ПЕТЛЕВОГО ПРАЙМЕРА

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Сальмонеллез остается одной из ведущих причин бактериальных кишечных инфекций человека и животных. Молекулярная диагностика радикально трансформировала диагностический ландшафт сальмонеллезной инфекции, однако остается времени- и ресурсозатратной. Изотермическая амплификация ДНК, например, петлевая изотермальная амплификация (LAMP), выполняемая при постоянной температуре, является основой разработки быстрых диагностических тестов, которые могут быть адаптированы к формату point-of-care (PoC) и выполнены в условиях ограниченных ресурсов и вдали от централизованных лабораторий. Целью исследования было разработать и валидировать новый метод детекции *Salmonella enterica* в образцах кала человека методом LAMP, в котором мониторинг результатов амплификации достигается с помощью петлевого флуоресцентно-меченного праймера, несущего также внутренний гаситель флуоресценции. Предложенный метод имеет предел обнаружения (LoD 95%) 250 копий на реакцию, чувствительность 86,84% (95% ДИ: 71,91–95,59%) и специфичность — 96,49% (95% ДИ: 87,89–99,57%) относительно qPCR, а также показал большую устойчивость к ингибиторам амплификации ДНК в образцах фекалий. Введение разных флуоресцентных меток в петлевые праймеры для FLP-LAMP с разными мишенями потенциально обеспечивает возможность мультиплексирования и одновременного выявления нескольких патогенов, что увеличивает диагностические возможности изотермальной амплификации.

Ключевые слова: *Salmonella enterica*, LAMP, петлевой флуоресцентный праймер, флуоресцентный зонд, аналитическая валидация, ингибиторы ДНК

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Salmonellosis is one of the most common anthropozoonotic infections caused by Gram-negative bacteria of the genus *Salmonella*, predominantly *Salmonella enterica* subsp. enterica [1]. According to the World Health Organization (WHO), 93–200 million cases of salmonellosis leading to acute gastroenteritis are registered annually, and about 155,000 cases worldwide result in death [2]. Therefore, salmonellosis remains a major public health problem in both developed and developing countries.

Diagnosis of salmonellosis includes the analysis of epidemiological and epizootological data, clinical signs, and pathomorphological changes, which can support a preliminary diagnosis. A definitive diagnosis is established using laboratory methods. *In vivo* diagnostics typically relies on serological, molecular genetic, and microbiological approaches.

In current clinical practice, the gold standard for detecting salmonellosis is microbiological culture, i.e., bacteriological inoculation of patient specimens onto selective media (e.g., *Rappaport–Vassiliadis medium*) [3]. The main drawbacks are the long turnaround time (48–96 hours), which delays timely initiation of targeted therapy, and reduced sensitivity if the patient has received antibiotics before sample collection. Microbiological testing requires dedicated facilities, equipment, and highly trained personnel, has low throughput, and is poorly suited for mass screening and epidemiological surveillance.

Rapid diagnostic methods include allele-specific real-time PCR and ELISA. Quantitative allele-specific PCR can shorten time to result (2–4 hours) and improve analytical sensitivity to 1–10 colony-forming units per milliliter (CFU/mL) [4]. PCR targets include *invA*, *ttr*, *bcfD*, and other conserved *Salmonella*-specific sequences [5]. However, the method is limited in many regions due to expensive equipment (real-time thermocyclers), insufficient personnel qualification in clinical diagnostic laboratories, and relatively high test cost.

Methods of isothermal amplification, in particular loop-mediated isothermal amplification (LAMP) developed by Notomi et al., have become a promising solution for point-of-care diagnostics. LAMP amplifies DNA at a constant temperature of 60–65 °C without thermal cycling, using a thermostable DNA polymerase (e.g., the large fragment of DNA polymerase I from *Geobacillus stearothermophilus*) and a highly specific set of four (less often six) primers recognizing six (or eight) distinct sites within the target DNA. This confers exceptionally high specificity of targeted sequence amplification [6]. Compared with PCR/qPCR, LAMP offers high specificity, rapid amplification (20–30 minutes), increased tolerance to amplification inhibitors in clinical samples, and does not require expensive instrumentation [7]. Various LAMP modifications are used in instrument-free tests or integrated into portable point-of-care devices for field use [8].

Multiple approaches have been proposed to visualize and monitor LAMP results, including turbidimetry, colorimetric assays, intercalating fluorescent dyes (SYTO, SYBR Green I), and modified oligonucleotides carrying fluorophores (primer-probes, molecular beacons, hybridization probes) [9]. The latter approach is particularly attractive due to multiplexing potential via multiple probes labeled with different dyes (FAM, HEX, ROX, Cy5) [8]. Nevertheless, the use of fluorescently labeled loop primers with an internal quencher in LAMP remains underexplored.

Here, we developed and validated a new approach to generate a real-time fluorescent signal in LAMP using a fluorescently labeled loop primer that also carries an internal fluorescence quencher, using diagnostic amplification of a fragment of the *Salmonella enterica bcfD* gene as a model. We hypothesized that loop primer-probes would enable reliable real-time monitoring comparable to intercalating dyes while opening opportunities for future multiplexing.

METHODS

Preparation of Control Material

A 211-nucleotide fragment of the *bcfD* gene (NC_003197, *Salmonella enterica* subsp. enterica) was amplified from *Salmonella* genomic DNA using primers bcfD-F3 and bcfD-B3 and cloned into a vector using the Quick-TA kit (Evrogen, Russia) following the manufacturer's instructions. Plasmid clone structure was confirmed by Sanger sequencing using the BigDye Terminator 3.1 kit (Applied Biosystems, USA) on an ABI 3730 genetic analyzer (Applied Biosystems, USA) at the Molecular Diagnostics Laboratory of ICBFM SB RAS.

The control plasmid (pBCFD) was linearized with BamHI and quantified using the Spectra Q BR kit (Sesana, Russia). The linearized plasmid was serially diluted over a range of 10^0–10^6 copies in buffer containing 10 mM Tris-HCl (pH 8.0), 2.5 μg/mL yeast RNA, and 0.01% NaN3. Copy numbers in dilutions were determined by droplet digital PCR (ddPCR) on the QX200 Droplet Digital PCR system (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions using primers Slm1 and Slm2 (600 nM) and the fluorescent probe Slm-PF (200 nM).

LAMP

Standard 20 μL LAMP reactions contained: 1× Gss-Sto polymerase buffer (40 mM Tris-HCl pH 8.9; 10 mM $(NH_4)2SO_4$; 10 mM KCl; 8 mM $MgSO_4$; 2.5% DMSO; 0.1% Triton X-100), 1.5 mM each dNTP, 0.4 μM outer primers (F3/B3), 0.3 μM loop primers (LF/LB), 1.6 μM inner primers (FIP/BIP), DNA template, and 3 units of Gss-Sto polymerase (ICBFM SB RAS). For fluorescence monitoring, either fluorescently labeled loop primers (SLB-LB1F, SLB-LB2F, SLF-LB1H) or the intercalating dye SYTO-13 at 0.5 μM were used. LAMP was performed in a CFX96 Touch thermocycler (Bio-Rad, Hercules, CA, USA) using 120 cycles of amplification at 60 °C with fluorescence acquisition in the HEX/FAM channel every 15–30 seconds.

Specificity was assessed by electrophoresis in 1.8% agarose gel to verify the characteristic LAMP amplicon pattern or its absence in the no-template control (data not shown). Amplification results were evaluated using the Tt (time-to-threshold) parameter, defined as the time required for the fluorescence curve to cross a threshold line set at the mean plus three standard deviations of the negative control.

Real-Time PCR (qPCR)

qPCR reactions (20 μL) contained: 1× PCR buffer (64 mM Tris-HCl pH 8.9; 16 mM $(NH_4)2SO_4$; 0.05% Tween-20; 3 mM $MgCl_2$; 0.002% NaN_3), 0.6 μM forward and reverse primers and 0.15 μM fluorescent probe (Table 1), 2 units of Taq polymerase (SibEnzyme, Russia), and DNA template. Cycling conditions were: Taq activation at 96 °C for 15 min; then 45 cycles of 95 °C for 10 s and 60 °C for 40 s with fluorescence acquisition in the FAM and Cy5 channels. Data were analyzed in CFX Manager (Bio-Rad).

Limit of Detection, Clinical Sensitivity and Specificity

The limit of detection (LoD 95%) was evaluated by varying control plasmid concentration: 65, 125, 250, 500, and 1000 copies per reaction, with 20 technical replicates at each concentration. LoD95% was defined as the concentration at which a positive reaction (Tt of negative control minus Tt of

Table 1. Oligonucleotide primers and fluorescent probes

Name	Sequence (5'–3')
bcfD-FIP	GCACTTTACCGGTACGCTGAATACAGCGGCAATTTCAACCA
bcfD-BIP	CGGTCTGGATTCGCAGGTCAAAGCGATAGCCTGGGGAAC
bcfD-F3	CCGGACAAACGATTCTGGTA
bcfD-B3	CCGACATCGGCATTATCCG
bcfD-LF	TACCCCCTCCGGCTTTTG
bcfD-LB	ACAATGCGTCTTATCGCTACG
SLF-RB1H	HEX-TTTTTTACCCCC(T-BHQ1)CCGGCTTTTG
SLB-LB1F	FAM-AAAAACAATGCGTC(T-BHQ1)TATCGCTACG
SLB-LB2F	FAM-ACAATGCGTCT(T-BHQ1)ATCGCTACG
Slm1	GGCAATTTCAACCATGCAGGC
Slm2	CCAGACCGCTGCACTTTACCG
Slm-PF	FAM-AGCCGGAGGGGGTACGAGCG-BHQ1

sample > 10 min) was observed in at least 95% of technical replicates.

Clinical sensitivity and specificity were evaluated on 95 DNA samples from children's stool collected at G. N. Speransky Children's City Clinical Hospital No. 9. qPCR targeting the *bcfD* gene was used as the reference standard. Samples were considered qPCR-positive if the Cq value was less than 34, corresponding to the system's LoD95% of ≤ 12.5 copies per reaction.

RESULTS

Design and Optimization of a Fluorescently Labeled Loop Primer

Development of the LAMP visualization method using fluorescently labeled loop primers was based on a previously published, well-characterized set of LAMP primers specific to a bcfD gene fragment. We used a concept analogous to TaqMan-like probes that generate fluorescence through hybridization rather than enzymatic hydrolysis [10]. In the free state, such a probe forms a three-dimensional conformation that brings a fluorophore and a quencher into proximity, quenching fluorescence via FRET. Upon hybridization to a complementary target sequence, the probe becomes linear, increasing the distance between the fluorophore and the quencher and increasing fluorescence.

Based on this principle, we synthesized a series of fluorescently labeled loop primer-probes for LAMP (Table 1). Each oligonucleotide contained: (1) a fluorophore (FAM or HEX) at the 5' end; (2) a free 3' OH-end available for elongation; and (3) an internal BHQ1 quencher introduced via a thymidine residue (T-BHQ1) located near the 3' end.

The structures of the conventional loop primers bcfD-LF and bcfD-LB served as starting points for design of the labelled probes. For three oligonucleotides, the distance between the fluorophore and quencher was varied: five deoxyadenosines (5 dA) were added to the 5' end of SLB-LB1F, and five deoxythymidines (5 dT) were added to the 5' end of SLF-LB1H to increase the FRET-pair spacing. The third variant (SLB-LB2F) carried the fluorophore directly at the native 5' end without extension.

Comparison of Fluorescent Loop Primer-Probes

Replacing a conventional loop primer (LF or LB) with a fluorescently labeled loop primer-probe (0.3 μM) in a LAMP reaction using pBCFD template (3000 copies per reaction) produced a clear fluorescence increase curve dependent on the oligonucleotide variant (Fig. 1). All three primer-probe variants generated fluorescence upon incorporation into the growing amplicon.

Signal amplitude was similar across various probes; however, the oligonucleotide with increased fluorophore–

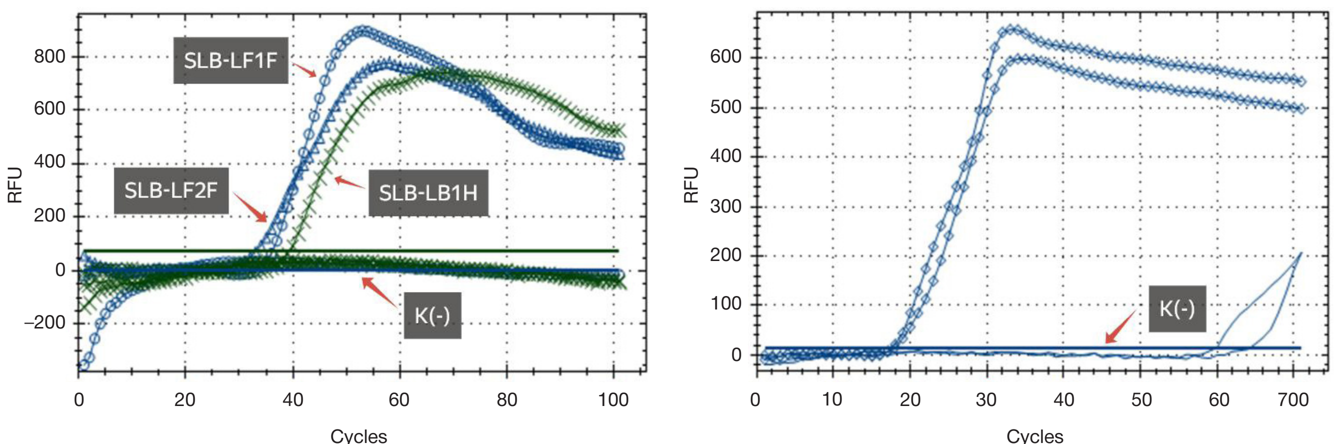

Fig. 1. LAMP fluorescent curves. The left panel depicts various fluorescently-labelled loop primers; the right panel demonstrates results of LAMP with the SYTO-13 intercalating dye

quencher spacing (SLB-LB1F) showed a higher signal amplitude (~35–40% of baseline fluorescence). As expected, using the intercalating dye SYTO-13 yielded a substantially shorter Tt (~9–10 min) compared with the fluorescent loop primer-probes (~16–19 min), likely due to the much larger number of dye molecules binding to the amplicon.

Increasing the concentration of the SLF-LB1H loop primer-probe from 0.3 to 0.6 μM did not substantially affect Tt but markedly improved signal amplitude from ~10–15% to 15–35% of the baseline fluorescence prior to amplification (Fig. 2). Higher concentrations did not notably increase the fluorescence amplitude; therefore, 0.6 μM was selected for subsequent experiments.

Determination of the Limit of Detection

The LoD95% is a key analytical characteristic defining the minimum amount of target DNA that can be reliably detected. We evaluated LoD for both visualization approaches by titrating DNA template at 65, 125, 250, 500, and 1000 copies per reaction (20 replicates per concentration). For the bcfD-LAMP-SLF-LB1H system, LoD95% was 250 copies per reaction (19/20 replicates positive; Tt = 20.67 ± 2.60 min). In comparison, SYTO-13 produced a slightly lower LoD: 125 copies per reaction (20/20 replicates; Tt = 17.78 ± 2.07 min), corresponding to approximately a twofold difference in sensitivity between the two visualization methods.

A critical parameter for diagnostic LAMP is the assay duration, because amplification time is constrained by the risk of nonspecific product formation in no-template controls (NTC). Prolonged incubation increases the risk of false-positive results and incorrect clinical interpretation. Because no fluorescence increase was observed in negative controls up to 60 minutes or longer, and the largest Tt for positive samples at LoD95% did not exceed 25 minutes for bcfD-LAMP-SLF-LB1H, the diagnostic LAMP duration was set to 30 minutes.

Testing on Clinical Samples

To determine clinical sensitivity and specificity of bcfD-LAMP-SLF-LB1H, 95 DNA samples from children's stool were analyzed. qPCR targeting bcfD with an established LoD95% of ≤ 12.5 copies per reaction (Cq ~34) was used as the reference standard. Samples were considered qPCR-positive if Cq < 34.

Clinical sensitivity of LAMP-SLF-LB1H was 86.84% (95% CI: 71.91–95.59%) and specificity was 96.49% (95% CI: 87.89–99.57%) (Tables 2, 3). For comparison, SYTO-13 yielded higher sensitivity (94.74%) with the same specificity. Cohen's kappa, a measure of agreement between two classification systems, was 0.844 (95% CI: 0.734–0.955) for LAMP-SLF-LB1H versus qPCR, indicating good agreement. For LAMP-SYTO13, kappa was higher (0.912; 95% CI: 0.828–0.996), also reflecting good agreement.

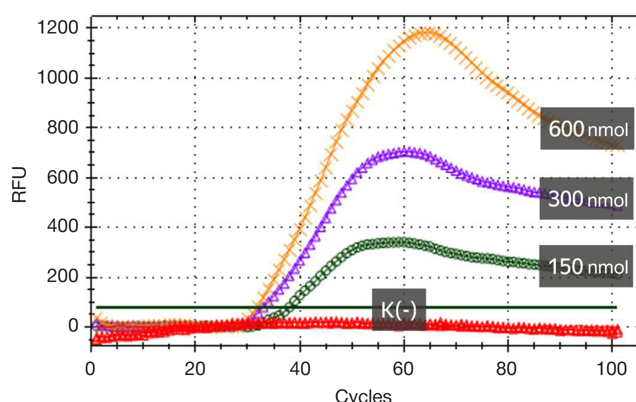

Fig. 2. Influence of a loop probe-primer SLF-LB1H concentration on the fluorescence amplitude

Discordant results and inhibitor tolerance

The main reason for discrepancies between LAMP-SLF-LB1H and qPCR was the difference in LoD: the method using fluorescent loop primers has a higher LoD (i.e., lower sensitivity) than qPCR. Analysis of discordant samples (qPCR-positive, LAMP-negative) showed Cq values in the 31–33 range, close to the positivity threshold of 34, suggesting borderline low amounts of target DNA.

To investigate discordance, two samples that were LAMP-SLF-LB1H-positive but negative in the initial qPCR analysis were retested by qPCR after fivefold dilution of DNA. Dilution is commonly used to reduce the concentration of amplification inhibitors that may be present in stool. After dilution, both samples became qPCR-positive with borderline Cq values (32–33), further supporting greater tolerance of LAMP-SLF-LB1H to fecal DNA amplification inhibitors. This is consistent with the well-known increased resistance of LAMP to inhibitors such as hemoglobin, bilirubin, heme, and other stool components.

DISCUSSION

Interest to isothermal amplification methods such as LAMP continues to grow because of their potential for decentralized diagnostics. Several approaches to generate fluorescent signal in LAMP using modified oligonucleotides have been proposed, including molecular beacons, hybridization probes (TaqMan-like probes operating via hybridization without cleavage), intercalating dyes (SYBR Green, SYTO), and fluorescent nucleotides (FL-dNTPs) incorporated during synthesis [11].

In this study, we developed and evaluated a LAMP test with fluorescent signal detection using fluorescently labeled loop primer-probes that are directly incorporated into the growing amplicon. The key advantage of this approach is that each incorporated primer-probe molecule contributes to

Table 2. Results of testing 95 children's stool DNA samples for *Salmonella* Comparison of LAMP-SLF-LB1H vs qPCR

	qPCR (+)	qPCR (–)	N
LAMP-SLF-LB1H (+)	33	2	35
LAMP-SLF-LB1H (–)	5	55	60
N	38	57	
	qPCR (+)	qPCR (–)	N
LAMP-SYTO13 (+)	36	2	38
LAMP-SYTO13 (–)	2	55	57
N	38	57	

Table 3. Diagnostic performance of LAMP methods compared with qPCR

Metric	LAMP-SLF-LB1H	LAMP-SYTO13
Sensitivity	86.84% (95% CI: 71.91–95.59%)	94.74% (95% CI: 82.25–99.36%)
Specificity	96.49% (95% CI: 87.89–99.57%)	96.49% (95% CI: 87.89–99.57%)
Cohen's kappa	0.844 (95% CI: 0.734–0.955)	0.912 (95% CI: 0.828–0.996)
Positive predictive value	94.29%	94.74%
Negative predictive value	91.67%	96.49%

fluorescence in proportion to the amount of product formed. When a fluorescent loop primer is incorporated into amplicon concatemers, the oligonucleotide becomes linear, substantially increasing the distance between the fluorophore (5' end) and the quencher (T-BHQ1), which restores fluorescence. Increasing fluorophore–quencher distance by adding extra nucleotides (5 dA or 5 dT) increased signal amplitude, although the effect was moderate.

The closest analogue to our system is FLOS-LAMP (fluorescence of loop primer upon self-dequenching) [12, 13]. In that approach, a fluorophore is introduced within the 3′ region of the loop primer in a specific nucleotide context, leading to quenching in the unbound state and increased fluorescence upon incorporation into the amplicon duplex. Our primer design is considerably simpler.

On control samples with known DNA concentrations, LAMP-SLF-LB1H demonstrated LoD95% of 250 copies per reaction, which is twofold higher than LAMP-SYTO13 (125 copies per reaction). This difference is expected because many more intercalating dye molecules bind to the amplicon and generate a stronger fluorescence signal. Nevertheless, LoD95% of 250 copies per reaction remains acceptable for clinical diagnostics, since typical *Salmonella* loads in stool samples from patients with acute gastroenteritis substantially exceed this value [14]. Further optimization of amplification conditions may improve sensitivity and reduce assay time. On a panel of 95 clinical stool DNA samples, LAMP-SLF-LB1H achieved sensitivity of 86.84% and specificity of 96.49% relative to qPCR.

Two samples that were LAMP-SLF-LB1H-positive but initially qPCR-negative became qPCR-positive after dilution, indicating the presence of amplification inhibitors. Isothermal amplification, including LAMP, is known for increased tolerance to inhibitors present in clinical samples [7]. The Gss-Sto DNA polymerase used here contains a DNA-binding domain and shows higher inhibitor tolerance than native enzymes [15]. The observed higher resistance of LAMP-SLF-LB1H to inhibitors compared with qPCR is consistent with the literature and is practically relevant, as it can reduce sample preparation requirements and enable less labor-intensive DNA extraction protocols. This observation should be confirmed more rigorously in larger studies.

A potential advantage of using fluorescently labeled loop primers is the ability to use primers with different fluorophores (FAM, HEX, ROX, Cy5) for simultaneous detection of multiple targets in a single LAMP reaction. This is especially important for mixed infections or panel tests detecting multiple pathogens (e.g., *Salmonella*, *Shigella*, *Vibrio*). A multiplex LAMP system using fluorescent loop primers could serve as the basis for a robust point-of-care diagnostic platform for rapid detection of multiple pathogens [16, 17].

Study limitations include the relatively small clinical panel size for robust diagnostic validation. In addition, although *bcfD* is considered *Salmonella*-specific, cross-testing against other closely related Enterobacteriaceae and intestinal pathogens is required. The study used high-quality instrumentation (CFX96 Touch, Bio-Rad), which may not reflect real-world PoC settings. Finally, intensive optimization was intentionally avoided to assess the reproducibility of the concept under routine LAMP conditions.

Despite these limitations, the results demonstrate the potential of fluorescent loop primers as a visualization system for LAMP. The method can be adapted to portable fluorescence devices for PoC diagnostics. The possibility of using different fluorophores opens prospects for multiplex test development and integration into molecular diagnostic devices for resource-limited settings.

CONCLUSIONS

We developed and validated a new LAMP design using a fluorescently labeled loop primer-probe that generates a real-time fluorescent signal for detection of Salmonella enterica based on the bcfD gene. The method provides acceptable analytical characteristics: LoD95% = 250 copies per reaction, sensitivity of 86.84% (95% CI: 71.91–95.59%) and specificity of 96.49% (95% CI: 87.89–99.57%) compared with qPCR. Using fluorescent loop primers labeled with different fluorophores opens prospects for multiplex LAMP to simultaneously detect several pathogens in a single reaction.

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