RESEARCH ARTICLE

Comparison of PCR and Loop Mediated Isothermal Amplification Method for Detection of *Salmonella* spp. in Chicken Meat

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Abstract

The aim of the study was to compare Polymerase Chain Reaction (PCR) and Loop Mediated Isothermal Amplification (LAMP) assay for detection of *Salmonella in* chicken meat. Food safety remains a major concern worldwide, with outbreaks of foodborne illness resulting in significant costs to individuals and to the food industry. The *Salmonella* genus is the most prevalent group of foodborne pathogens that are regularly isolated from food-producing animals and thus are one of the leading causes of food borne diseases. Out of total 40 *Salmonella* isolates, LAMP and PCR assay detected 36 (90%) and 32 (80%) isolates, respectively. The sensitivity of the LAMP assay was noted to be 10-fold higher than that of PCR whereas the specificity of both LAMP and PCR was same.

Key words: Food, LAMP, Meat, PCR.

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Introduction

Salmonella organisms are responsible for a variety of acute and chronic diseases in poultry, animals and humans (Barrow *et al*., 2012) and is a common cause of mortality and morbidity due to water and food borne infections in almost all countries causing human gastroenteritis and typhoid fever (Malorny *et al*., 2008). Transmission of Salmonella is often associated with animals (CDC, 2007) and plant products (CDC, 2009). Foods of animal origin and contaminated poultry products (eggs and poultry meat) were the main carriers of *Salmonella* infection (Cosby *et al*., 2015).

Salmonella is a member of the Enterobacteriaceae family and is Gram-negative, rod-shaped, facultatively anaerobic bacteria. It is a ubiquitous and hardy bacterium that can survive several weeks in a dry environment and several months in water. Genus *Salmonella* consists of two species namely *Salmonella enterica* and *Salmonella bongori*. More than 2600 serovars of *S. enterica* have been identified so far, and many of these serovars can infect both humans and animals (Chlebicz and Slizewska, 2018). Salmonellosis is caused by Non-Typhoidal *Salmonella* (NTS) *enterica* serotype which includes *Salmonella enterica* serotype Enteritidis and *Salmonella enterica* serotype Typhimurium, the two most important serotypes transmitted from animals to humans. The disease has a self-limiting gastroenteritis syndrome which is manifested as diarrhoea, fever, and abdominal pain with an incubation period of 4-72 h (Crump *et al*., 2015). Conventional *Salmonella* detection is usually performed using a culture-based method, which is time-consuming, labour intensive, and unsuitable for on-site testing and high-throughput analysis. To date, there are many detection methods with a unique detection system available for *Salmonella* utilizing immunological techniques (ELISA), molecular techniques (PCR), mass spectrometry, spectroscopy, optical phenotyping, loop-mediated isothermal amplification (LAMP), biosensor methods etc. (Awang *et al*., 2021). These methods can identify and discriminate Salmonella down to their serotype level (Ferone *et al*., 2020).

PCR has been successfully identified as a valuable method that provides rapid, sensitive, and accurate detection of selected genes of targeted pathogens (Germini *et al*., 2009). Despite the benefits, PCR method requires costly equipment such as thermocycler for DNA amplification and electrophoresis units that makes it unsuitable for use in field conditions. Hence, there was a need for developing diagnostic methods which could overcome the limitations of PCR and can potentially be used on-site.

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Notomi *et al*. (2000) developed a new method of nucleic acid amplification called Loop Mediated Isothermal Amplification (LAMP) which is simple in operation, low cost, and high sensitivity as well as specificity (Shao *et al*., 2011). This novel method, under isothermal conditions (60-65°C), can amplify a few copies of DNA to 10 copies in less than an hour. The LAMP technique would be most appropriate for laboratory detection and is well adopted as a field level diagnostic test in developing countries (Boehme *et al*., 2007; Rekha *et al*., 2014). Therefore the present study was undertaken with the objective to compare PCR and LAMP method of detection of *Salmonella* in chicken meat.

Materials & Methods

Sample Collection

Total 40 *Salmonella* isolates obtained after cultural isolation and biochemical characterization in chicken meat were used in this study. Pure colonies of isolates were inoculated on nutrient agar for further study.

DNA Extraction

The DNA from the *Salmonella* isolates was obtained by heat and lysis method of DNA extraction (Dashti *et al*., 2009). This DNA was then used as template for performing PCR and LAMP assay.

Polymerase Chain Reaction

The isolates were screened for the presence of *invA* gene by PCR as described by Kumar *et al*. (2008). The primer sequence for target gene *invA* was F: GTG AAA TTA TCG CCA CGT TCG GGC AA; R: TCA TCG CAC CGT CAA AGG AAC C, with product size of 284 bp. The reaction mixture was prepared in 200 µL thin-walled PCR tubes. Total 25 µL reaction mixture consisted of 12.5 µL 2X PCR master mix, 1 µL each of forward and reverse primer (10 pmol of each primer), 5.5 µL nuclease free water and 5 µL of DNA template. The amplification was performed in thermal cycler, and cycling conditions for *inv*A gene primer were initial denaturation at 94°C for 7 min followed by 35 cycles of denaturation at 94°C for 45 s, amplification at 51°C for 45 s, and extension at 72°C for 45 s and final extension at 72°C for 7 min. On completion of the reaction the final amplified products were analysed by agarose gel

Table 1: Description of primer used for confirmation of *Salmonella* spp. by LAMP

Sr. No.	Target Gene	Primer sequence (5′ → 3′)		Reference
1.	*inv*A	*inv*A-F3	GAACGTGTCGCGGAAGTC	Ge *et al.* (2019)
		*inv*A-B3	CGGCAATAGCGTCACCTT	
		*inv*A-LF	TCAAATCGGCATCAATACTCATCTG	
		*inv*A-LB	AAAGGGAAAGCCAGCTTTACG	
		*inv*A-FIP	GCGCGGCATCCGCATCAATATCTGGATGGTATGCCCGG	
		*inv*A-BIP	GCGAACGGCGAAGCGTACTGTCGCACCGTCAAAGGAAC	

F3: Forward Outer Primer; B3: Backward Outer Primer; LF: Loop Primer Forward; LB: Loop Primer Backward; FIP: Forward Inner Primer; BIP: Backward Inner Primer.

Table 2: Quantity and concentration of various components used for LAMP

Sr. No.	Components	Quantity Final	Concentration
1	Isothermal Amplification buffer	2.50 µL	1 X
2	dNTP Mix	3.50 µL	1.20 mM
3	MgSO4	1.50 µL	6 mM
4	Primer *inv*A-F3	2.00 µL	0.20 µM
5	Primer *inv*A-B3	2.00 µL	0.20 µM
6	Primer *inv*A-FIP	1.80 µL	1.60 µM
7	Primer *inv*A-BIP	1.80 µL	1.60 µM
8	Primer *inv*A-LF	0.50 µL	0.40 µM
9	Primer *inv*A-BF	0.50 µL	0.40 µM
10	Nuclease Free Water	4.9 µL	--
11	*Bst* Polymerase	1 µL	8 Units/ µL
TOTAL		**22.00** µL	--
12	DNA Template	3.00 µL	--
GRAND TOTAL		**25.00** µL	**--**

electrophoresis on 2% agarose gel. The gel was visualized under UV transilluminator, and results were documented in gel documentation system.

Loop Mediated Isothermal Amplification

The optimization of LAMP assay was carried out as described by Pavankumar *et al*. (2014) by conducting the assay at different temperatures (58°C, 60°C, 62°C, 63°C, 65°C and 66°C) and time periods (50 min, 60 min and 70 min). The LAMP reaction mixture was optimized using different concentrations of inner primers, outer primers, loop primers, $MgSO_4$ and dNTPs. In all reactions, 8 units of *Bst* polymerase and 3 µL of DNA template were added. Significant visual turbifity and fluorescence was obtained at 65°C and 60 min on addition of SYBR green dye, henceforth the same was used in subsequent reactions. The reaction mixture was incubated at 65°C for 60 min in water bath and then heated to 80°C for 2 min to terminate the reaction. Primers used in LAMP assay are given in Table 1 and the quantity and concentration of various components used is mentioned in Table 2.The results of LAMP assay were visualized by performing agarose gel electrophoresis and by addition of SYBR green dye to the LAMP products.

Assessment of Sensitivity by LAMP and PCR

The DNA of *Salmonella* isolates was extracted by boiling method (Lee *et al*., 2009). Concentration of DNA was checked using nanodrop 1000. The DNA was diluted up to 100 ng/µL, 10 ng/µL, 1 ng/µL, 100 pg/µL, 10 pg/µL and 1 pg/µL. The sensitivity (detection limit) of LAMP and PCR assay was evaluated using 3 µL of DNA from each dilution which was taken as a template to perform *Salmonella* specific LAMP and PCR, making the resultant concentrations of 300 ng/tube, 30 ng/tube, 3 ng/tube, 300 pg/tube, 30 pg/tube and 3 pg/tube. After completion of both methods with DNA templates as described above, the results were analysed by addition of SYBR green dye and performing agarose gel electrophoresis.

Assessment of Specificity by LAMP and PCR

DNA of *Salmonella* spp., *Staphylococcus aureus*, *Bacillus cereus*, *Klebsiella* spp. and *Escherichia coli* was used as template to perform LAMP and PCR with *Salmonella* specific primers. On completion of the reaction the amplified products were analysed by agarose gel electrophoresis. The gel was visualized under UV transilluminator in gel documentation system.

Results and Discussion

Polymerase Chain Reaction

Out of total 40 isolates, 32 (80%) were confirmed as *Salmonella* by targeting *inv*A gene (Fig. 1), which were in accordance with the findings of Kaushik *et al*. (2014) who reported 76.92% (50/65) detection rate of *Salmonella* from the isolates, whereas lower detection rate of 6.60% and 55% was reported by Abdel-Aziz (2016) and Sharma and Das (2016), respectively. The low detection rate may be due to good hygienic practices. On the contrary high detection rate of 92.86% and 89% from the isolates was reported by Naik *et al*. (2015) and Jayaweera *et al*. (2020).

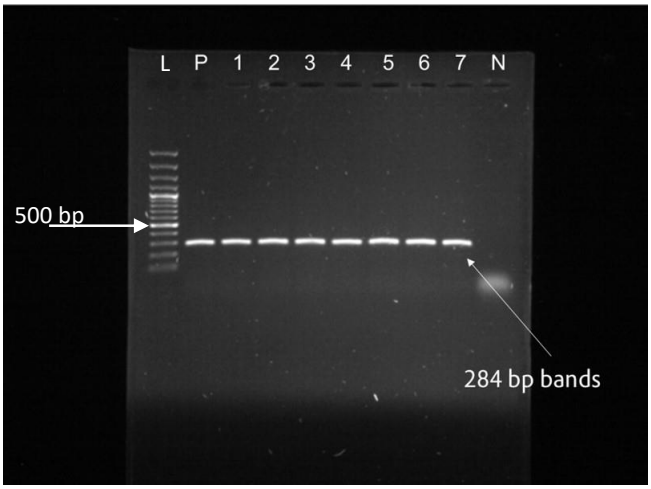

Fig. 1: Agarose gel showing amplification product of *inv*Agene (284 bp) L: 100 bp DNA ladder; P: Positive control; Lane 1-7: Positive samples; N: Negative control

Loop Mediated Isothermal Amplification

In the present study (Fig. 2) LAMP assay showed 36 isolates out of 40 (90%) confirmed *Salmonella* spp. Almost similar results were given by Ohtsuka *et al*. (2005) and Zhang *et al*. (2018) who found 90.90% and 93.55% detection rate respectively of *Salmonella* spp. by LAMP. Various workers (Pavankumar *et al*., 2014; Zhuang *et al*., 2014; and Priya *et al*., 2020) reported 100% prevalence of *Salmonella* using LAMP.

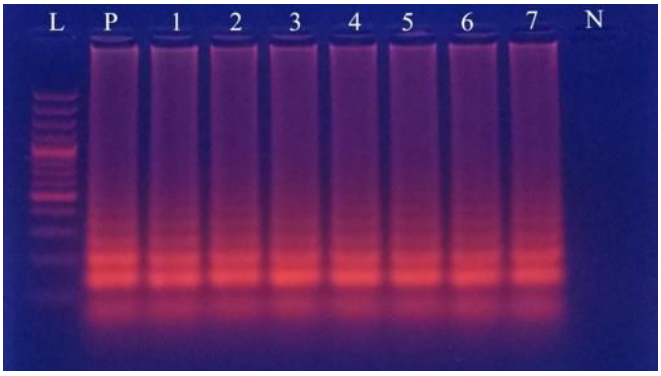

Fig. 2: Ladder like pattern of LAMP products on 2% Agarose gel L: 100 bp DNA Ladder; P: Positive control; Lane 1-7: Positive *Salmonella* isolates; N: Negative control

Comparison of Sensitivity by Assessment of LAMP and PCR

LAMP detected DNA concentrations of 300 ng/tube, 30 ng/tube and 3 ng/tube (Fig. 3). The PCR detected DNA concentrations of 300 ng/tube and 30 ng/tube (Fig. 4).

Results indicated that LAMP had 10 folds greater sensitivity than PCR which was similar to the results of Hara-Kudo *et al.* (2005), Zhuang *et al.* (2014), and Priya *et al*. (2020). In contrast to these findings 100 folds greater sensitivity of LAMP than PCR was reported by Pavankumar *et al.* (2014).

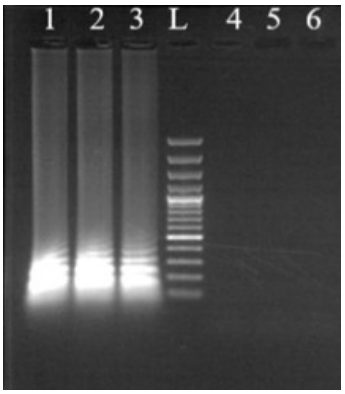

Fig. 3: LAMP assay sensitivity by electrophoresis
LAMP carried out at different concentrations of DNA
L: Ladder; Lane 1-3: LAMP reactions at 300 ng, 30 ng, 3 ng of template DNA; Lane 4-6 LAMP reactions at 300 pg, 30 pg and 3 pg of template DNA

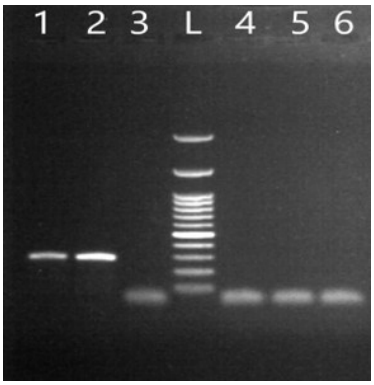

Fig. 4: PCR assay sensitivity by electrophoresis
PCR carried out at different DNA concentrations
L: 100 bp DNA Ladder; 1: 30 ng/tube; 2: 300 ng/tube; 3: 3 ng/tube; 4: 300 pg/tube; 5: 30 pg/tube; 6: 3 pg/tube

Comparison of Specificity by Assessment of LAMP and PCR

Both LAMP and PCR showed 100% specificity (Fig. 6 and Fig. 7). The results of the present study corroborate with the results of Pavankumar *et al.* (2014), Zhuang *et al.* (2014), and Priya *et al*. (2020).

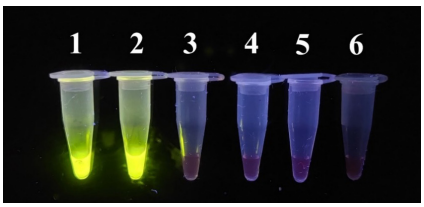

Fig. 5: LAMP assay specificity by addition of SYBR green

Tube 1: Positive control, Tube 2: *Salmonella* spp. Tube 3: *Staphylococcus aureus,* Tube 4: *Bacillus cereus,* Tube 5: *Klebsiella* spp. and Tube 6: *Escherichia coli*

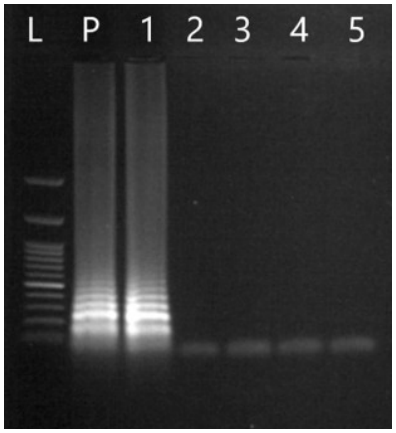

Fig. 6: LAMP assay specificity by electrophoresis
LAMP reaction with different bacterial DNA template
L: 100bp DNA Ladder; P: Positive control; 1-*Salmonella* spp.; 2-*Staphylococcus aureus*;3-*Bacillus cereus;* 4-*Klebsiella* spp.; 5-*Escherichia coli*

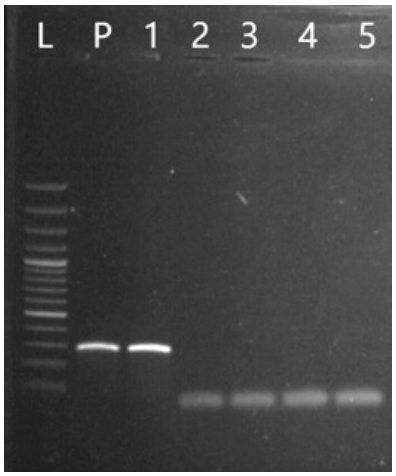

Fig. 7: PCR assay specificity by electrophoresis

PCR reaction with different bacterial DNA template
L: 100 bp DNA Ladder; P: Positive control; 1: *Salmonella* spp.; 2: *Staphylococcus aureus;* 3: *Bacillus cereus;* 4: *Klebsiella* spp.; 5: *Escherichia coli*

Conclusion

The PCR technique by targeting *inv*A gene detected 80% (32/40) isolates as *Salmonella* spp. whereas with LAMP assay the detection rate was 90% (36/40). Both PCR and LAMP were able to successfully detect *Salmonella* spp. and did not give any false positive result with non-*Salmonella* strains. Thus, the specificity of both LAMP and PCR was found to be 100%. the sensitivity of the LAMP assay was noted to be 10-fold higher than that of PCR. LAMP assays could help in robust detection of *Salmonella* spp. with reliable sensitivity and specificity.

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