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Rapid Detection of *Salmonella* spp. in Animal Origin Foods by In-House Developed Loop-Mediated Isothermal Amplification (LAMP) Assay

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A B S T R A C T

Salmonellosis is one of the most common causal food borne disease in India. An in-house loop-mediated isothermal amplification (LAMP) reaction was established and evaluated for sensitivity and specificity in detecting the presence of *Salmonella* spp. isolates in foods of animal origin from Mumbai, India. The aim of this study was to develop in-house LAMP for simple and inexpensive detection of *Salmonella* spp. in animal origin foods using specifically designed primers targeting *inv*A gene contains sequences unique to this genus. The reaction was optimized using genomic DNA of *S. typhimurium* (MTCC 3224) as the template. The assay was conducted in a water bath for 1h at 65°C. The results were visualized after addition of SYBR Green® fluorescent dye. The test was further evaluated on 59 serotyped *Salmonella* field isolates to ensure its reliability and usefulness. The results were compared with those obtained by gold standard culture method and Polymerase Chain Reaction (PCR). This method was highly specific and 10 times more sensitive in detecting *Salmonella* spp. compared to the optimized conventional polymerase chain reaction (PCR) method. This simple method may be applied on field diagnostic laboratories without access to expensive equipment.

Introduction

Salmonella is a gram-negative bacterium belonging to family Enterobacteriaceae and is an important cause of food-borne bacterial infections in both developed and developing countries (European Food Safety Authority, 2007). A variety of foods have been implicated as vehicles transmitting salmonellosis to humans, including poultry, beef, pork, eggs, milk, cheese, fish, shellfish, fresh fruit and juice, and vegetables (Kshirsagar *et al.*, 2014). *Salmonella* was the second most common aetiology, causing 1,449 (18%) of the 7,998 outbreaks with a confirmed or suspected single aetiology and 39,126 (19%) illnesses in the United States (Gould *et al.*, 2013).

At present, standard methods for *Salmonella* detection and identification include mainly bacteria isolation and biochemical identification. Culture-based methods are reliable but laboratory-intensive, time-consuming and demanding several days for definitive results, while cross biochemical reaction can occur between different species under Enterobacteriaceae (Yang *et al.*, 2010;

Kokkinos *et al.,* 2014). For public health and the food industry rapid, sensitive and specific method to detect *Salmonella* in foods is required (Ueda and Kuwabara, 2009). A number of nucleic acid-based molecular methods have been successfully used to detect *Salmonella* spp. (Zhuang *et al.,* 2014) and the requirement for expensive equipment's and reagents renders them unfavorable for wide-scale use, particularly under field conditions (Kokkinos *et al.,* 2014).

Loop-mediated Isothermal Amplification (LAMP) was developed by Notomi *et al.,* (2000), which can be used to detect food-borne pathogens. It amplifies target gene under isothermal conditions with high efficiency, specificity and rapidity (Hara-Kudo *et al.,* 2005). This method relies on the auto-cycling strand displacement nature of *Bst* DNA polymerase with high strand displacement activity and a set of two specially designed inner and two outer primers. This novel method can amplify a few copies of DNA to 10^9 copies in less than an hour under isothermal conditions (60–65°C) (Zhuang *et al.,* 2014). The expensive equipment like thermocycler is not necessary to give a high level of precision, equivalent or greater, when compared to PCR. The LAMP technique would be most suited for out-of-laboratory detection activities and would also be most suited for poorly equipped laboratories or testing institutions (Boehme *et al.,* 2007).

The *inv*A gene of *Salmonella* contains sequences unique to this genus and has been proved as a suitable PCR target, with potential diagnostic applications (Rahn *et al.,* 1992). In an International research project for the validation and standardization of PCR for the detection of five major food-borne pathogens including *Salmonella*, the most selective primer set which targets the location at 139-141 on genomic sequence of *Salmonella*, signified the *inv*A gene. In present research work, we developed and applied a specific in-house LAMP method for detecting *Salmonella* spp. from foods of animal origin and the results were compared with gold standard culture methods and PCR assay.

Materials and Methods

Isolation of *Salmonella* spp. from animal origin foods

A total of 490 different animal origin foods comprising of *viz*. meat (50 muttons, 150 chicken, 80 buffalo meat and 60 pork), and 50 each of fish, eggs and milk were collected from various retail shops from different regions of Mumbai City. These samples were further processed for isolation of *Salmonella* spp. following standard technique as per IS 5887 (Part 3): 1999. Out of 490 animal origin food samples, 59 (12.04%) samples showed presence of *Salmonella* as shown in Table 1.

All of these isolates were further characterized by biochemical tests and the results were interpreted and validated as per Bacteriological Analytical Manual for *Salmonella* (2007). Further, these 59 positive *Salmonella* isolates were then subjected for detection by standardized conventional Polymerase Chain Reaction (PCR) and Loop-mediated Isothermal Amplification (LAMP) methods.

Bacterial strains and DNA extraction

Five reference strains including reference strain of *S. typhimurium* (MTCC 3224) and 04 other non-*Salmonella* species *viz*. *E. coli*. (MTCC-443), *Pseudomonas aeruginosa* (MTCC-4673), *Shigella flexneri* (MTCC-1457) and *Staphylococcus aureus* (MTCC 3160) were used in this study procured from MTCC, Institute of Microbial Technology,

Chandigarh, India. Additionally, 59 field isolates of *Salmonella* spp. isolated and serotyped at National *Salmonella* Centre (Veterinary), Division of Bacteriology and Mycology, Indian Veterinary Research Institute (IVRI), Izatnagar, Bareilly, India were included in the study.

Genomic DNA of *Salmonella* spp. was extracted as per the protocol of Rawool *et al.*, (2007) with slight modifications. A colony of *Salmonella* isolate on Nutrient agar was picked and mixed with 1000 µl of NSS in centrifuge tube. It was then centrifuged at 10,000 rpm for 10 min. After centrifugation, the pellet formed was dissolved in 100 µl of nuclease free water (NFW), vortexed and further boiled at 100^0C for 10 min. The centrifuge tube was subjected to rapid cooling in ice which was followed by centrifugation at 10,000 rpm for 10 min. Then, the upper aqueous phase which contained DNA was transferred to sterile micro-centrifuge tube. These extracted DNAs were further used for amplification. Until use these were stored at freezing temperature (-20^0 to -80^0C) in sterile micro-centrifuge tube.

LAMP primer design

The primers were designed using online LAMP primer designing software Primer Explorer V4 program by Eiken Chemicals Co. Ltd., Japan. The gene sequences were aligned and analysed by the online software program (http://primerexplorer.jp/elamp4.0.0/index.
html) to design the following *Salmonella*-specific primers: F3 (forward outer primer), B3 (backward outer primer), FIP (forward inner primer) and BIP (backward inner primer). The outer primers consisted of F3 and B3, while the inner primers consisted of FIP and BIP and were commercially synthesized by Integrated DNA Technologies (IDT) obtained from Sigma Aldrich, Bangalore, India. The primers used in PCR for the specific detection of *Salmonella* spp. were previously described by (Rahn *et al.*, 1992). The sequences of the primers are summarized in table 2.

Optimization of LAMP assay

To optimize LAMP conditions, the test was carried at different temperatures (60^0C, 62^0C, 64^0C, 66^0C, 68^0C, and 70^0C) and also at different time periods (40, 50, 60, and 70 min). LAMP reaction mixture was optimized using different concentrations of inner primers, outer primers, MgSO4, and dNTPs. In all reactions, eight units of *Bst* DNA polymerase, 1 M betaine and 2 µl of DNA template were used. The LAMP product was examined by direct observation of the reaction tubes. The insoluble white precipitate was noticed in the reaction tube due to formation of magnesium pyrophosphate, which had direct correlation with amplification. The LAMP products were also examined by submarine gel electrophoresis on 2% agarose gel with prestained ethidium bromide (5 mg/mL) in 0.5X tris-EDTA electrophoresis buffer. One microliter of SYBR green dye in 1:100 dilutions was used as colouring agent to the LAMP product and observed under day light.

Optimization of *invA* PCR

The PCR procedure to screen isolates for *inv*A gene (*Salmonella* genus specific) was standardized as described by Rahn *et al.*, (1992) with some modifications. The amplification was performed in total volume of 25 µl containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs mix, 10 pmol each of forward and reverse primers, 5 µl of template DNA and 1 unit Taq DNA polymerase. The cycling condition comprised an initial denaturation at 95^0C for 5 min, followed by 30 cycles each of denaturation at 95^0C for 1 min, primer

annealing at 65^{0}C for 1 min, elongation at 72^{0}C for 1 min and finally a single extension step at 72^{0}C for 10 min. Amplified PCR product (284 bp) was analyzed by agarose gel electrophoresis on 1.5% agarose gel.

Sensitivity of the LAMP assay

For sensitivity (detection limit) of LAMP assay was evaluated using 200, 150,100 and 50 ng/µl DNA. The DNA was further diluted serially 10 folds to 2, 1.5, 1 and 0.5 pg/tube, respectively. 2 µl of DNA from each dilution was taken to perform *Salmonella* specific LAMP, making the resultant minimum concentrations of 4, 3, 2 and 1pg/tube DNA, respectively. The reaction was performed at 65^{0}C for 60 min, and the results of this assay were compared with conventional PCR.

Specificity of LAMP assay

The specificity of LAMP assay was tested using standard *Salmonella* DNA template and 4 other templates from non-*Salmonella* strain. The DNA templates were prepared as described previously. The specificity of *Salmonella* specific LAMP was performed by testing it with four other bacterial species viz. *E. coli*, *Pseudomonas aeruginosa*, *Shigella flexneri* and *Staphylococcus aureus.* The reaction was performed at 65°C for 60 min and the results of this assay were compared with conventional PCR.

Results and Discussion

LAMP conditions

In order to determine the optimal conditions of LAMP, *S. typhimurium* reference strain (MTCC 3224) was used as the target template. LAMP assays were incubated under isothermal condition between 60 and 70^{0}C. The presence of significant visual turbidity and fluorescence on addition of SYBR green dye was observed at 65.0^{0}C (Figures 1A & B). The optimal LAMP duration time was 60 min longer incubation up to 105 min had no influence on the final results. Therefore, the final LAMP condition for all strains was 65^{0}C for 60 min then 80^{0}C for 2 min to deactivate the polymerase. After completion of LAMP reaction, amplified DNA were analyzed on 2% of agarose gel by electrophoresis at 90V for 45 min. A 100bp DNA ladder was also loaded along with LAMP products and subsequently observed under U.V. Transilluminator of gel documentation system which exhibited specific ladder like pattern in case of DNA amplification (Figure 1C). The PCR was standardized for *inv*A gene (284 bp) using reference strain (Figure 1D).

Determination of detection limits (sensitivity) and specificity of LAMP

Sensitivity of LAMP

The sensitivity (detection limit) of LAMP assay for *Salmonella* spp. was evaluated using 200, 150, 100 and 50 ng/µl DNA. This DNA was diluted serially 10 folds to 2, 1.5, 1 and 0.5 pg/tube, respectively and 2µl of DNA from each dilution was taken as a template to perform *Salmonella* specific LAMP, making the resultant minimum concentrations of 4, 3, 2 and 1pg/tube DNA, respectively. The amplification was carried out at 65°C for 60 min. and reaction was terminated at 80°C for 2 min. The study showed that LAMP could detect up to 4 ng/tube concentration of DNA but further failed to detect 400pg/tube concentration of DNA (Figures 2 A & B) Thus, the LAMP could able to detect the *Salmonella* spp. up to 4ng/tube concentration of DNA.

Similar protocol of DNA dilution was adopted for evaluating sensitivity (detection limit) of conventional PCR assay. However, conventional PCR could be able to detect the

DNA up to 40 ng/tube of DNA, failing to detect any further dilutions. Thus, conventional PCR could able to detect the *Salmonella* spp. up to 40ng/tube concentration of DNA.

The sensitivity (detection limit) of the LAMP assay was noted to be 10 fold greater than that of conventional PCR as LAMP could detect 4 ng/tube of *Salmonella* spp. DNA concentration, whereas, conventional PCR could able to detect 40ng/tube of concentration of *Salmonella* spp. DNA (Figure 2C).

Specificity of LAMP

In the present study, the specificity of LAMP assay was tested using standard *Salmonella* spp. DNA template and 4 other templates from non-*Salmonella* strains viz. *E coli*, *Pseudomonas aeruginosa*, *Shigella flexneri* and *Staphylococcus aureus*. The LAMP was carried out as per the standard protocol at 65^0C for 60 min in water bath. It was found that the LAMP assay successfully amplified *Salmonella* spp. DNA only, while it did not amplify any non-*Salmonella* organisms. Similarly, the PCR detected *Salmonella* spp. successfully and did not give any positive result with non-*Salmonella* strains (Figures 3A and B). Thus, the specificity of both LAMP and conventional PCR was found to be 100%.

Analysis of field samples

Out of 490 various animal origin food samples, 59 (12.04%) showed presence of *Salmonella* spp by cultural isolation followed by serotyping. Amongst 50 muttons, 150 chicken, 80 buffalo meat, 60 pork, and 50 each of fish, eggs and milk, the *Salmonella* isolates recovered were 5, 24, 16, 9, 3, 0 and 2, respectively. Of the 59 *Salmonella* isolates 22 (37.28%) belonged to *S. Dublin*, 13 (22.03%) *S. typhimurium* and 12 (20.33%) isolates each belonged to *S. enteritidis* and Other *Salmonella* spp. respectively as shown in Table 3.

After subjecting all the 59 positive *Salmonella* isolates to LAMP, it was observed that all the *Salmonella* isolates positive by cultural methods were detected positive by (100%) using LAMP technique. However, on screening by conventional PCR, it was observed that PCR was able to detect 58 (98.30%) positive *Salmonella* isolates. The only negative sample observed by PCR was the *Salmonella* spp. isolated from fish.

Table.1 Details of samples positive for *Salmonella* spp

Sr. No.	Type of food sample	Number of samples examined	Number of *Salmonella* isolates recovered	Prevalence (%)
1.	Mutton	50	05	10
2.	Chicken	150	24	16
3.	Buffalo meat	80	16	20
4.	Pork	60	09	15
5.	Fish	50	03	06
6.	Eggs	50	00	-
7.	Milk	50	02	04
Total		**490**	**59**	**12.04**

Table.2 Oligonucleotide sequences of LAMP primers used in this study

Sr. No.	Target gene	Primer	Sequence (5'-3')
1.	*inv*A	**LAMP Primers** **F3:** Forward outer primer **B3:** Backward outer primer **FIP:** Forward inner primer (F1c-F2) **BIP:** Backward inner primer (B1c-B2) **PCR Primers**	 **F3:** GAA CGT GTC GCG GAA GTC **B3:** CGG CAA TAG CGT CAC CTT **FIP:** GCG CGG CAT CCG CAT CAA TAT CTG GAT GGT ATG CCC GG **BIP:** GAA CGG CGA AGC GTA CTG GAC ATC GCA CCG TCA AAG GAA **F:** GTGAAATTATCGCCACGTTCGGGCAA **R:** TCATCGCACCGTCAAAGGAACC

Table.3 Results of field samples by culture, PCR and LAMP

Sr. no	Source of sample	*inv*A Positive			Serotypes
		Cultural	PCR	LAMP	
1	Mutton	05	05	05	*S.* Enteritidis, *S.* Dublin, Other *Salmonella*
2	Chicken	24	24	24	*S. typhimurium*, *S.* Enteritidis, *S.* Dublin, Other *Salmonella*
3	Buffalo meat	16	16	16	*S.* Dublin, Other *Salmonella*
4	Pork	09	09	09	*S.* Dublin, *S. typhimurium*, *S.* Enteritidis
5	Fish	03	02	03	*S.* Enteritidis, Other *Salmonella*
6	Milk	02	02	02	*S.* Dublin
Total		**59**	**58**	**59**	**22** *S.* Dublin, **13** *S. typhimurium*, **12** *S.* Enteritidis and **12** Other *Salmonella*

Figure.1 (A) Turbidity due to formation of magnesium pyrophosphate
Tube 1-No turbidity Tube 2– Turbidity
(B) Addition of SYBR green dye
Tube 1- No fluorescence indicating no DNA amplification
Tube 2– SYBR green fluorescence indicating DNA amplification

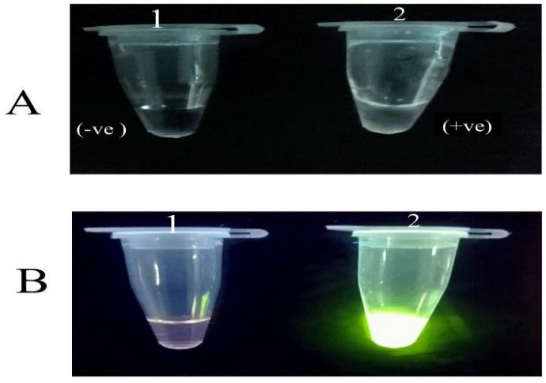


Figure.1 (C) Ladder like pattern of LAMP products
Lane 1-3: Ladder like pattern of LAMP products of *Salmonella*;
Lane 4: Negative control; Lane M: Marker
(D) Standardization of PCR for *invA* gene of *Salmonella* spp.
Lane 1-3: PCR products of *Salmonella*, spp. Lane M: Marker

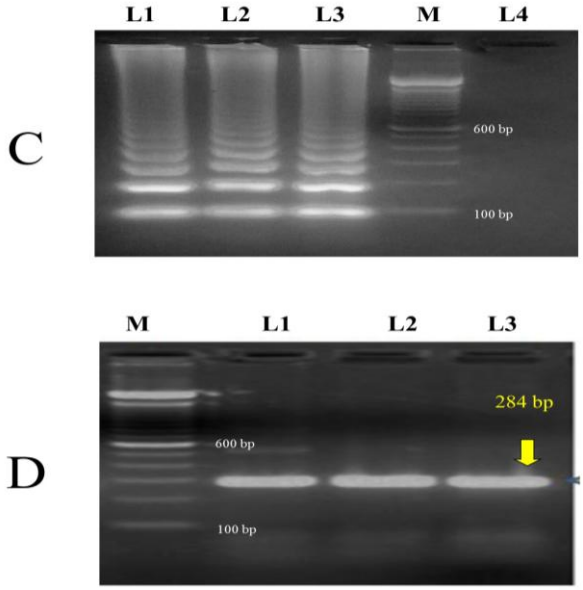


Figure 2: (A) Sensitivity of LAMP assay by addition of SYBR green dye;
Tube 1-3: 400, 40 and 4 ng and in Tube 4-6: 400, 40 and
4 pg per tube of DNA
(B) Ladder like Pattern of LAMP products at different concentrations of DNA
(C) Sensitivity of PCR; Lane 1-3: PCR reactions at 400, 40, 4 ng and in
Lane 4-6: 400, 40 and 4 pg per tube of DNA, Lane M:100bp DNA Ladder

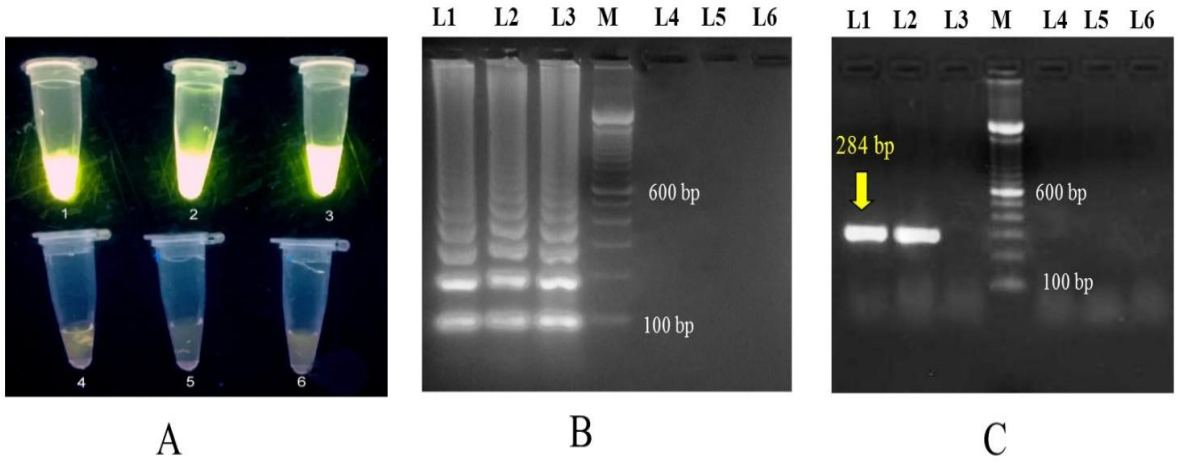
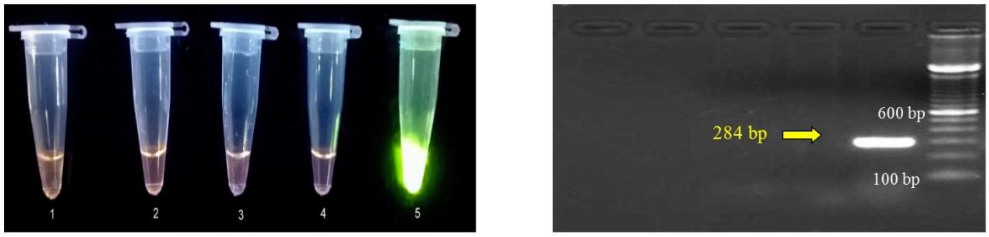


Figure.3(A) Specificity of LAMP assay for *Salmonella* spp.; Tube 1-4: No any green Fluorescence observed for *E. coli*, *P. aeruginosa*, *Shigella flexneri* and *S. aureus.* Tube 5: Green fluorescence for *Salmonella* spp. **(B)** Specificity of PCR for *Salmonella* spp. Lane 1-4: No DNA amplification Observed for *E. coli*, *P. aeruginosa*, *Shigella flexneri* and *S. aureus* Tube 5: DNA amplification for *Salmonella* spp.; Lane M:100bp DNA Ladder



A B

In the present study, an in-house LAMP method was developed and successfully applied it to detect *Salmonella* spp. in foods of animal origin. The target gene *invA* contains sequences unique to this genus *Salmonella* and encodes a protein in the inner membrane of bacteria which is responsible for invasion to the epithelial cells of the host (Darwin and Miller, 1999).

All of the 59 isolates of *Salmonella* spp. recovered from foods of animal origin were found positive (100%) using LAMP technique. The similar findings were also reported by Hara-Kudo *et al.,* (2005) who could detect all the 220 strains of *Salmonella* spp. (100%) using LAMP technique. However, Zhang *et al.,* (2012) detected only 93.55% (29 of 31) positive *Salmonella* isolates analyzed by LAMP. This may be attributed to variations in the reaction mixture and primers used lowering the sensitivity of detection.

Compared with PCR could detect 98.30% (58 out of 59) while LAMP could detect 100% (59 out of 59) of *Salmonella* isolates. This may be attributed to the presence of four specific primers targeting six distinct sites on the *inv*A gene. However, Ohtsuka *et al.,* (2005) and Tang *et al.,* (2012) reported 90% and 72.72% detection of positive *Salmonella* isolates by PCR, respectively, while LAMP technique successfully identified all the *Salmonella* spp. analyzed (100%). The sensitivity (detection limit) of the LAMP assay was noted to be 10 fold greater than that of conventional PCR as LAMP could detect 4 ng/tube of *Salmonella* spp. DNA concentration, whereas, conventional PCR could able to detect 40ng/tube of concentration of *Salmonella* spp. DNA.

The results are in accordance with a study conducted by Ueda *et al.,* (2009) using LAMP assay for detection of *Salmonella* spp. in food and human samples who reported that the LAMP could detect 10^2 CFU/ml whereas, the PCR could detect 10^3-10^5 CFU/ml of *Salmonella* spp. indicating that LAMP was 10 times more sensitive than PCR in their experiment. The findings are similar with Tang *et al.,* (2012), Zhang *et al.,* (2012) and Wang and Wang *et al.,* (2013) who reported the sensitivity of LAMP assay 10 times higher than the PCR-based method. The results are also in accordance with Abdullah *et al.,* (2014) who reported that LAMP method was highly specific and 10 times more sensitive in detecting *S. typhi* as compared to the optimized conventional PCR method. However, Pavan Kumar *et al.,* (2014) reported that the LAMP test developed for *S. typhimurium* was 100 times more sensitive than conventional PCR. Similarly, Chan *et al.,* (2015) studied detection limit for LAMP assay for *Salmonella* spp. was 10^1 CFU/ml whereas, PCR could detect 10^3 CFU/ml of *Salmonella* spp. by targeting *Inv*E gene. This variation may be attributed to the difference in LAMP

conditions and due to species level LAMP for *Salmonella* spp. carried out in their study.

In the current study the specificity of both LAMP and conventional PCR was found to be 100%. The findings in the present study are similar with the studies carried out by Hara-Kudo *et al.*, (2005), Ueda *et al.,* (2009), Zhao *et al.,* (2010) and Chan *et al.,* (2015) who reported the specificity of LAMP was equally significant as that of PCR assay i.e. 100%. The specificity results (100%) observed in present study are also in accordance with Zhuang *et al.,* (2014) who reported that LAMP technique could amplify all the 44 *Salmonella* strains successfully, but none of 9 non-*Salmonella* standard strains used under study.

The study also revealed that LAMP could differentiate and specifically detect only *Salmonella* spp. from other 4 non-*Salmonella* strains. However, both LAMP and PCR assays were successfully able to identify only *Salmonella* spp. without giving any false positive results for non- *Salmonella* strains showing 100% specificity for both the assays.

In conclusions *Salmonella* spp. is one of the major food-borne pathogen that causes outbreak of food-borne disease around the world. Salmonellosis is endemic in India and thus, there is need to develop a rapid, sensitive and specific test for its detection. Therefore, the in-house developed LAMP assay has higher sensitivity compared to PCR. Moreover, this LAMP assay can further increase the scanning speed and save more time. From a practical point of view, this in-house developed LAMP is a suitable, cost effective method for resource-limited laboratories and in field applications.

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