Research Article

Qualitative Method of *Salmonella* Screening, Identification and Confirmation in Shrimp Matrices using Loop-mediated Isothermal Amplification Method, Enzyme Linked Immuno Fluorescent Assay and Standard Conventional Method

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ABSTRACT

Contamination due to *Salmonella* in seafood processing have become greatest threat today. Effective monitoring and testing methodology used to get highest recovery of *Salmonella* to conform the requirements of Hazard Analysis Critical Control Point is a greatest challenge in today's world. These challenges can be effectively addressed if the conventional detection methods which are labor-intensive and time-consuming shall be replaced by more rapid and highly sensitive methods. Study was doneto identify natural seafood contamination by *Salmonella* on raw freshly harvested *Litopenaeus vannamei* shrimp matrices and individually quick frozen *Litopenaeus vannamei* shrimp matrices. Study showed fastest recovery and accuracy with LAMP methodology (3M™ MDS System) than with ELFA (Bioemerieux- Vidas SPT) and Convectional method.

Key words: LAMP (Loop Mediated Isothermal Amplification) Method, ELFA (Enzyme Linked Immuno fluorescence assay, USFDA (The United States Food and Drug Administration, Salmonella, 3M MDS system (Molecular detection system), HACCP (Hazard Analysis Critical Control Point).

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INTRODUCTION

Microbial contamination of food is a major concern of consumers, industries and regulatory authorities worldwide. Contamination of seafood with *Salmonella* is a major concern worldwide. While seafood have identified a huge niche worldwide with huge volume of export especially in India. With increase in global population and seafood consumption, aquaculture practices have also increased drastically and majority of the aquaculture shrimps are being exported to USA from India. Shrimp is identified as highly economical and internationally traded seafood.[1] In recent years due to the increased food-borne sickness, round the globe many regulatory bodies have fixed zero tolerance level to some of the food-borne pathogens like *Salmonella* spp., *Vibrio cholerae*, *V. parahaemolyticus*, and *V. vulnificus* in any seafood export consignment.[2]

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Salmonella is a Gram-negative, usually motile, facultative anaerobic, flagellated rod-shaped. *Salmonella enteritidis* has become the most common cause of salmonellosis which is the second major cause of foodborne disease acquired in the United States and leads episodes of hospitalization and death.[3,4] Conventional culture methods are always considered as Golden standards for the isolation and identification of foodborne pathogens, but the conventional methods are time consuming as it involves cultures on selective media and characterization of suspicious colonies by biochemical tests followed by serotyping, labor consuming and chances of human error are high as the differentiation of typical and atypical colony characteristics on selective

agar plates[5] increases the bulkiness of manual labor before inferring on the contamination status. Hence, these conventional techniques for monitoring of critical control points (CCPs) in hazard analysis critical control point (HACCP) format are cumbersome, where huge number of samples are to be analyzed, and the result is required within a very short time.

Immunological methods detect unique *Salmonella* molecules using two antibodies; a surface-bound primary antibody to capture the target molecule and a reporter antibody to detect the antibody target complex. Immunological techniques can replace isolation agars, lowering the time to presumptive positive result on a day. *Salmonella* detection are also done using Enzyme-linked fluorescent assay (ELFA). ELFA are more complex and incorporate washes between capture and reporter steps to remove non-target molecules that cause false positive results.

Rapid, sensitive method LAMP (loop-mediated isothermal amplification), have been developed and applied in the detection and identification of Salmonella in foods[6] and have been reviewed comprehensively (Yang *et al*., 2018). Distinct advantages of LAMP over other methodologies are that it runs at constant temperature (Notomi *et al*., 2000) and it have high tolerance to matrix inhibitors (Kaneko *et al*., 2007).

MATERIALS

Apparatus

Blender and sterile blender jars, beakers, conical flask, sterile sample bags, glass rods, calibrated weighing balance, circulating water bath, Sterile Petri dishes, Inoculating needle and inoculating loop, Vortex mixer, large scissors, scalpel, and forceps, bunsen burner, pH meter, Cotton, Swabs, Vidas, 3M Molecular detection system, Biosafety cabinet, Laminar Air flow, Dry heat incubator for incubating lysis tubes of MDS 2 assay, Heat and Go incubator for incubation of vidas SPT test strips.

Medias and reagents

Lactose broth, Buffered Peptone water, Tetrathionate (TT) broth, Rappaport-Vassiliadis (RV) medium, Xylose lysine desoxycholate (XLD) agar, Hektoen enteric (HE) agar, Bismuth sulfite (BS) agar, Salmonella Chrom agar (Rambach agar), SPT- Vidas Biomerieux, Salmonella Supplement Biomerieux, 3M-Molecular detection Assay 2 *Salmonella* test kit, API NaCl 0.85 % for making suspension of organism, API 20E test strips of Biomerieux, API software for detection of organism.

Study Design

500 samples were collected and subjected to *Salmonella* analysis by three different method, Conventional method as per Bacteriological Analytical Manual Chapter 5[7,8] Enzyme Linked Immuno Fluorescence Assay using Vidas, Loop Mediated Isothermal Amplification method using 3M-MDS assay system. Out of 500 samples, 300 samples were raw freshly caught *Litopenaeus vannamei* shrimp, 150 samples were Raw peeled deveined tail on Individually quick frozen *Litopenaeus vannamei* shrimp and 50 samples were cooked peeled deveined tail on Individually quick frozen *Litopenaeus vannamei* shrimp. Isolation, identification and confirmation of *Salmonella* was performed as per BAM chapter 5-D.[7]

Also to check cross reactivity, 10 samples were inoculated with *Proteus,* 10 samples with *Enterobacter* and another 10 samples were inoculated with *Citrobacter*.[9]

Sample preparation adopted in the study

Sample preparation was done in different methods for cooked and raw shrimp. 30 subunits of cooked shrimp were collected as per BAM Ch.1; A – 1 (a) (Food Category II. - Foods that would not normally be subjected to a process lethal to *Salmonella* between the time of sampling and consumption). From this two composite samples of 375 g sample were analyzed. While for Canada consignments 125g one composite sample or 5 samples of 25 g were analyzed. When samples were screened as positive they were subjected to confirmatory test as per BAM Ch.5

15 subunits of raw shrimps were collected as per BAM Ch.1; A – 1 (a) (Food Category III. - Foods that would normally be subjected to a process lethal to *Salmonella* between the time of sampling and consumption). While for Canada- 5 samples, each sample of 25 g or 125 g composite (15 bags were selected and 25 g were taken from every 3 bags to make the sample volume as 125 g. Sample were run either as individual 5 sample or as composite 125 g). One composite sample of 375 g sample was analyzed. While for Canada consignments 125g one composite sample or 5 samples of 25 g was analyzed. In case if it was screened as positive the samples were subjected to confirmatory test as per BAM Ch.5.

Presumptive identification of *Salmonella* using LAMP, ELFA and Conventional method

LAMP method (Using 3M Molecular Detection System)- AOAC.OMA 2016.01: Samples were diluted as 1:9 dilution as per BAM Ch.1 by taking 375 g sample which was added into 3375 ml Buffered Peptone water.

This was homogenized at room temperature between 22- 25°C and humidity <65%. Samples were then incubated in the the pre-enrichment broth for 18-24 hr @37±1°C. After incubation time 20µl was transferred to lysis tube provided in test kit (in duplicate) which was placed on dry heat incubator for 15±1 min @100°C. Lysis tubes were then removed and kept for cooling for 15 min in cooling block. Again from this lysis tube 20µl was transferred to primer tube and read it in Molecular Detection System which gives results as positive or negative over the software.

ELFA method (Using Vidas, Biomerieux)- AOAC. OMA 2013.01: Samples were diluted as 1:9 dilution as per BAM Ch.1 by taking 375 g sample which was added into 3375 ml Buffered Peptone water. As per the test kit protocol 15 ml *Salmonella* supplement were also added. This was homogenized at room temperature between 22- 25°C and humidity <65%. Samples were then incubated in the pre-enrichment broth for 18-24 hr @41.5±1°C. After incubation time 500µl of the sample were trasnfetted to SPT test strip and placed on Vidas Heat and Go incubator for 5±1 min @131°C. Strps were then removed and left for cooling for 10 min. After this the strips were loaded on to the Vidas machine and read. Results will come as screened positive or screened negative within 48 min of placement of strip to machine. Tests were negative when Threshold value was less than 0.25 and test were positive when Threshold value was greater than 0.25.

Conventional method: Pre-enrichment of samples were done by adding 375g sample which was added into 3375 ml lactose broth. This pre-enrichment non selective Lactose broth containing samples were incubated for 24±2 hr at 35°C. For further enrichment 0.1ml of pre-enriched sample containing broth was transferred to 10 ml of Rappaport-Vassiliadis (RV) broth and 1ml was transferred to 10 ml of TetraThionate (TT) broth as per BAM Chapter 5. These tubes were then incubated as RV broth for 24hr at 42°C and TT broth for 24 hr 35°C ± 2. After incubation for another 24 hr samples from enrichment broths were sub-cultured by taking 3 loopful of broth to *Salmonella* selective medias bismuth sulfite (BS) agar, xylose lysine desoxycholate (XLD) agar, Hektoen enteric (HE) agar.

Isolation of Typical or Atypical *Salmonella* were done as per BAM Chapter 5

The enriched broths were mixed thoroughly using vortex or with rotating shakers and then streaked 3 mm loopful (10 µl) from BPW/RV/TTB on Salmonella Chrom agar or selective medias like bismuth sulfite (BS) agar, xylose lysine desoxycholate (XLD) agar, and Hektoen enteric (HE) agar. BS plates were prepared the day before streaking and stored in dark at room temperature until streaked. Pates were then incubated for 24 ± 2 hr at 35°C. Plates were examined for presence of typical or atypical colonies of *Salmonella.*

Characteristics of *Salmonella* (Typical /Atypical) were as follows

Typical colony characters appeared as Blue-green to blue colonies with or without black centers on Hektoen enteric (HE) agar, Pink colonies with or without black centers on Xylose lysine desoxycholate (XLD) agar, Brown, gray, or black colonies; sometimes they have a metallic sheen on Bismuth sulfite (BS) agar and pink colored colonies on Rambach *Salmonella* Chrom agar.

Atypical colonies appeared as yellow colonies with or without black centers on HE and XLD agars on HE and XLD agars., green colonies with little or no darkening of the surrounding medium on BS agar while green colored colonies on Rambach *Salmonella* Chrom agar.

Identification of *Salmonella*

Pure colonies were taken for biochemical identification system using API 20 E as per BAM Ch.5 E.9 and the organism was identified using API web software. Being fastidious organism one colony was selected for identification step. As a good laboratory practice number of colonies were selected based on size of colony.

RESULTS

Study was done by collecting 500 *Litopenaeus vannamei* shrimp samples, out of which 325 were freshly harvested and 175 were individually quick frozen. These were subjected to *Salmonella* analysis using three different methodologies like conventional method as per BAM Chapter 5 vs ELFA method vs LAMP method. Analysis were done to compare the effectiveness of test method and time required for analysis.

Out of 325 samples of raw freshly caught *Litopenaeus vannamei* shrimp, 42 samples were screened positive using LAMP method, 34 samples were positive by ELFA method and 23 samples were isolated with atypical and typical colonies over conventional method. The samples which were screened as positive were subjected to colony isolation and identification using API 20 E and API web software. The results showed that all 42 positive samples by LAMP method were confirmed as *Salmonella,* 25 positive samples by ELFA method were confirmed as *Salmonella* while remaining 9 positive samples by ELFA were confirmed as *Proteus, Enterobacter, Citrobacter, Cronobacter,* which were finalised as false positive. The 9 samples which were false

positive by ELFA were not-detected as positive neither by LAMP nor by conventional method. Conventional method showed 23 samples with atypical and typical colonies which were all confirmed as *Salmonella*.

From 175 samples of Raw Individually quick frozen shrimps analyzed 3% of the products were screened as contaminated with *Salmonella* when tested using LAMP only, which were confirmed as *Salmonella*. Though ELFA method detected 1% of samples as positive for *Salmonella* it were confirmed as false positive with *Enterobacter species*, while conventional method couldn't isolate typical or atypical colonies of *Salmonella* species.

Results showed that 13% of the freshly harvested shrimp were contaminated with *Salmonella* which were easily screened as positive using LAMP method within 24 hr when compared with ELFA and conventional method. Results showed that LAMP method was highly sensitive and specific for *Salmonella* detection as it is following high sensitive multi primer loop mediated nucleic acid amplification technology. Table 1 shows the comparative results for the detection and confirmation of *Salmonella* in freshly harvested *Litopenaeus vannamei* shrimp and Table 2 shows the results for confirmed samples of raw individually quick frozen shrimp. Cooked individually quick frozen were also tested which were detected as negative for *Salmonella*.

DISCUSSION

There is recurring *Salmonella* related food born infections from ingestion of food like fish, meat, crustaceans, vegetables, salads etc. *Salmonella* screening, identification, detection, confirmation is now very important for foods as the infections are even becoming fatal. Here 500 seafood samples were collected out of which 175 was individually quick frozen product and other 325 samples were raw freshly harvested *Litopenaeus vannamei* shrimp, these were analysed by 3 different methods like LAMP, ELFA and conventional culture method. Out of this about 13% of freshly harvested raw shrimp and 3% of frozen or processed shrimp were detected to be with *Salmonella* using LAMP technology, while ELFA method showed 7% raw and 1% frozen shrimp as positive with *Salmonella*, but conventional culture method couldn't screen any *Salmonella* in frozen shrimp though conventional method identified 7% positivity in freshly harvested shrimp. Conventional culture methods are usually based on nutrient acquisition, biochemical characteristic identification.[10] LAMP methodology took only 24 hr to have the screened report to be released though its confirmation took 48 hr, ELFA method though took only 24 hr for screening and 48 hr for confirmation due to false positivity during screening step affected the operational activities in food processing.

In this study, we used a rapid and simpler method proposed by Ferretti *et al*.[11] that relying on nonselective enrichment in Buffered Peptone Water followed by cell breaking and LAMP method to detect Salmonella spp. within a maximum of 18-24 hr from the receipt of food samples. The ability of LAMP method to detect a very low number of *Salmonella* cells in seafood, is proposing that this method can be used to generate quantitative data on *Salmonella* in seafood, facilitating the implementation of control measures for *Salmonella* contamination in seafood at harvest and post-harvest levels. The 18-24 hr pre-enrichment LAMP procedure could offer a rapid and good diagnostic tool for the routine monitoring of detection of Salmonella in food samples compared to the conventional culturing method. Other studies have also reported that the use of a nucleic acid assays were more sensitive than the culture method for detecting *Salmonella* in food, especially in seafood, poultry, meat, and poultry related products.[12-16]

LAMP method which was performed by using 3M™ Molecular detection Assay 2 *Salmonella* uses novel loop mediated isothermal amplification method coupled with bioluminescence for detection of *Salmonella*. Universally invA gene is used as a target for detection of *Salmonella* as it is involved in invasion of epithelial cell. 3M MDS-2 assay is also targeting invA gene detection. As it is genetic based results were identified as highly specific and sensitive, while false positivity were high with ELFA method because some microbes like *Enterobacter, Cronobacter, Citrobacter, Proteus* etc shows characters similar to *Salmonella*. False positivity with ELFA method is because the antibody coated in solid phase receptacle are made to bind with *Salmonella* O (somatic) and *Salmonella* H (flagellar) antigen while it as the O and H antigens of some other organisms like *Proteus, Citrobacter freundii, Cronobacter and Enterobacter hermanii*. This is because these antigen on the cell surface have similar immunological and biochemical properties with Salmonella species.[9] Conventional method as described by BAM is always a golden method while, the chances of human error for missing out the typical or atypical colonies were high, while recovery or identification is purely based on the nutrient acquisition, biochemical characteristics, and metabolic products unique to Salmonella spp, which itself takes upto 7 days for confirmation

Table 1: Report of *Salmonella i*n Raw freshly caught *Litopenaeus vannamei* Shrimp.

Sample number	Salmonella presumptive screening report- LAMP* (3M MDS-2** Assay)	Confirmation report- BAM Method (Chapter 5)	Salmonella presumptive screening report- ELFA*** (mini Vidas)	Confirmation report- BAM Method (Chapter 5)	Salmonella presumptive screening report- Conventional method (BAM Chapter 5)	Confirmation report- BAM**** Method (Chapter 5)
1	Positive	*Salmonella*	Positive	*Salmonella*	Typical colonies isolated	*Salmonella*
2	Positive	*Salmonella*	Positive	*Salmonella*	Typical colonies isolated	*Salmonella*
3	Positive	*Salmonella*	Positive	*Salmonella*	Typical colonies isolated	*Salmonella*
4	Positive	*Salmonella*	Positive	*Salmonella*	Typical colonies isolated	*Salmonella*
5	Positive	*Salmonella*	Positive	*Salmonella*	Typical colonies isolated	*Salmonella*
6	Positive	*Salmonella*	Positive	*Salmonella*	Typical colonies isolated	*Salmonella*
7	Positive	*Salmonella*	Positive	*Salmonella*	Typical colonies isolated	*Salmonella*
8	Positive	*Salmonella*	Positive	*Salmonella*	Typical colonies isolated	*Salmonella*
9	Positive	*Salmonella*	Positive	*Salmonella*	Typical colonies isolated	*Salmonella*
10	Positive	*Salmonella*	Positive	*Salmonella*	Typical colonies isolated	*Salmonella*
11	Positive	*Salmonella*	Positive	*Salmonella*	Typical colonies isolated	*Salmonella*
12	Positive	*Salmonella*	Positive	*Salmonella*	Typical colonies isolated	*Salmonella*
13	Positive	*Salmonella*	Positive	*Salmonella*	Typical colonies isolated	*Salmonella*
14	Positive	*Salmonella*	Positive	*Salmonella*	Typical colonies isolated	*Salmonella*
15	Positive	*Salmonella*	Positive	*Salmonella*	Atypical colonies isolated	*Salmonella*
16	Positive	*Salmonella*	Positive	*Salmonella*	Atypical colonies isolated	*Salmonella*
17	Positive	*Salmonella*	Positive	*Salmonella*	Atypical colonies isolated	*Salmonella*
18	Positive	*Salmonella*	Positive	*Salmonella*	Atypical colonies isolated	*Salmonella*
19	Positive	*Salmonella*	Positive	*Salmonella*	Atypical colonies isolated	*Salmonella*
20	Positive	*Salmonella*	Positive	*Salmonella*	Atypical colonies isolated	*Salmonella*
21	Positive	*Salmonella*	Positive	*Salmonella*	Atypical colonies isolated	*Salmonella*
22	Positive	*Salmonella*	Positive	*Salmonella*	Atypical colonies isolated	*Salmonella*
23	Positive	*Salmonella*	Positive	*Salmonella*	Atypical colonies isolated	*Salmonella*
24	Positive	*Salmonella*	Positive	*Salmonella*	Typical and atypical colonies not identified	-
25	Positive	*Salmonella*	Positive	*Salmonella*	Typical and atypical colonies not identified	-
26	Positive	*Salmonella*	Negative	-	Typical and atypical colonies not identified	-
27	Positive	*Salmonella*	Negative	-	Typical and atypical colonies not identified	-
28	Positive	*Salmonella*	Negative	-	Typical and atypical colonies not identified	-
29	Positive	*Salmonella*	Negative	-	Typical and atypical colonies not identified	-
30	Positive	*Salmonella*	Negative	-	Typical and atypical colonies not identified	-
31	Positive	*Salmonella*	Negative	-	Typical and atypical colonies not identified	-

Table 1: Cont'd.

Sample number	Salmonella presumptive screening report- LAMP* (3M MDS-2** Assay)	Confirmation report- BAM Method (Chapter 5)	Salmonella presumptive screening report- ELFA*** (mini Vidas)	Confirmation report- BAM Method (Chapter 5)	Salmonella presumptive screening report- Conventional method (BAM Chapter 5)	Confirmation report- BAM**** Method (Chapter 5)
32	Positive	*Salmonella*	Negative	-	Typical and atypical colonies not identified	-
33	Positive	*Salmonella*	Negative	-	Typical and atypical colonies not identified	-
34	Positive	*Salmonella*	Negative	-	Typical and atypical colonies not identified	-
35	Positive	*Salmonella*	Negative	-	Typical and atypical colonies not identified	-
36	Positive	*Salmonella*	Negative	-	Typical and atypical colonies not identified	-
37	Positive	*Salmonella*	Negative	-	Typical and atypical colonies not identified	-
38	Positive	*Salmonella*	Negative	-	Typical and atypical colonies not identified	-
39	Positive	*Salmonella*	Negative	-	Typical and atypical colonies not identified	-
40	Positive	*Salmonella*	Negative	-	Typical and atypical colonies not identified	-
41	Positive	*Salmonella*	Negative	-	Typical and atypical colonies not identified	-
42	Positive	*Salmonella*	Negative	-	Typical and atypical colonies not identified	-
43	Negative	-	Positive	*Proteus*	Typical and atypical colonies not identified	-
44	Negative	-	Positive	*Proteus*	Typical and atypical colonies not identified	-
45	Negative	-	Positive	*Cronobacter*	Typical and atypical colonies not identified	-
46	Negative	-	Positive	*Citrobacter*	Typical and atypical colonies not identified	-
47	Negative	-	Positive	*Citrobacter*	Typical and atypical colonies not identified	-
48	Negative	-	Positive	*Enterobacter*	Typical and atypical colonies not identified	-
49	Negative	-	Positive	*Enterobacter*	Typical and atypical colonies not identified	-
50	Negative	-	Positive	*Enterobacter*	Typical and atypical colonies not identified	-
51	Negative	-	Positive	*Enterobacter*	Typical and atypical colonies not identified	-

NB: *LAMP -Loop Mediated Isothermal Amplification Method, **MDS-2 iMolecular Detection Assay, ***ELFA-Enzyme linked immunofluorescence Assay, ****BAM- Bacteriological Analytical Manual

Table 2: Report of *Salmonella* in Raw IQF *Litopenaeus vannamei* Shrimp.

Sample number	Salmonella presumptive screening report- LAMP* (3M MDS-2** Assay)	Confirmation report- BAM Method (Chapter 5)	Salmonella presumptive screening report- ELFA*** (mini Vidas)	Confirmation report- BAM Method (Chapter 5)	Salmonella presumptive screening report- Conventional method (BAM Chapter 5)	Confirmation report- BAM**** Method (Chapter 5)
1	Positive	*Salmonella*	Positive	*Enterobacter*	Typical and atypical colonies not identified	-
2	Positive	*Salmonella*	Positive	*Enterobacter*	Typical and atypical colonies not identified	-
3	Positive	*Salmonella*	Negative	-	Typical and atypical colonies not identified	-
4	Positive	*Salmonella*	Negative	-	Typical and atypical colonies not identified	-
5	Positive	*Salmonella*	Negative	-	Typical and atypical colonies not identified	-

NB: *LAMP -Loop Mediated Isothermal Amplification Method, **MDS-2 iMolecular Detection Assay, ***ELFA-Enzyme linked immunofluorescence Assay, ****BAM-Bacteriological Analytical Manual, IQF- Individually Quick Frozen

CONCLUSION

Our findings indicated that the pre-enrichment followed by LAMP method was rapid, simple, cost effective and time saving method that allowed the detection of *Salmonella* spp. within a maximum of 24-48 hr from the receipt of food samples with high accuracy, sensitivity and specificity.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

CCP: Critical Control Point; **HACCP:** Hazard Analysis and Critical Control Point; **ELFA:** Enzyme Linked Immuno Fluoresent Assay; **LAMP:** Loop Mediated Isothermal Amplification; **MDS:** Molecular Detection System; **API:** Analytical Profile Index; **AOAC:** Association of Official Analytical Chemist; **OMA:** Official Method of Analysis; **BAM:** Bacteriological Analytical Manual; **RV:** Rappaport Vassiliadis; **TTB:** Tetrathionate Broth; **XLD:** Xylose Lysine Deoxycholate; **BS:** Bismuth sulphite; **HEA:** Hektoen Enteric Agar; **Spp:** Species.

SUMMARY

The study conducted on comparison of conventional BAM method versus rapid methods (like LAMP and ELFA) showed high sensitivity and specificity using LAMP method than with ELFA and BAM method. Kumar *et al,* Surendran *et al*, Thamburan *et al* have investigated the occurrence of multiple serovars and antibiotic resistant Salmonella in shrimp samples,[17] which is of high significance as Salmonella causes serious food borne illness. The application of PCR test methodology for rapid detection of Salmonella in different matrices like food including seafoods, agricultural, feeds, environmental surveillance samples[18-22] were done by different researchers while our study using LAMP with multi primers made it more sensitive, specific and rapid which helps in fastest corrective and preventive actions to be setup in place by the manufacturing industry.

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