Simultaneous detection of food safety hazards (*Salmonella enterica* and *Listeria monocytogenes*) in table eggs using multiplex PCR

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Abstract

Foods are analyzed for food safety hazards in order to comply with food safety standards. Among foodborne pathogens *Salmonella enterica* and *Listeria monocytogenes* are recognized as major foodborne pathogens of public health significance worldwide. In the present study, multiplex polymerase chain reaction (mPCR) was used to screen for *S. enterica* and *L. monocytogenes* in table eggs collected from different markets (840 eggs). Pooled egg samples categorized as commercial and backyard eggs based on the source of collection. Collected eggs were screened for *S. enterica* and *L. monocytogenes* using mPCR assays. Conserved regions viz. *invA* and *prfA* genes were targeted for the specific detection of *S. enterica* and *L. monocytogenes*, respectively. mPCR and conventional method showed same results, prevalence of *S. enterica* at 12.5 and 33.3 per cent in commercial and backyard eggs, respectively. While, *L. monocytogenes* was undetectable in commercial eggs; but, detected only in backyard table egg sample (8.3%). Present study indicated complete concordance between specific pre-enrichment mPCR and conventional cultural methods. Results of the study underscored mPCR as steadfast rapid tool for the screening of table eggs for listed food safety hazards *S. enterica* and *L. monocytogenes* in table eggs.

Keywords: Eggs; Listeria; mPCR; Public Health; Salmonella

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Introduction

Salmonella enterica and *Listeria monocytogenes* are the major food safety hazards of public health concern often implicated in foodborne disease outbreaks (Singh *et al.*, 2012). Salmonellosis, caused by *Salmonella enterica*, a disease of economic significance having implications on the food industry and it is primarily associated with poultry and poultry products. Genus *Salmonella* comprises of over 2,600 serovars and it is the second largest cause of food poisoning in the world (Gal-Mor *et al.*, 2014); Salmonellae can also be transmitted from animals to humans through food; occasionally, person to person transmission also occurs through the faeco-oral route in addition to prevalent sporadic cases and it also cause severe extra-intestinal infections such as bacteremia and meningitis (Borges *et al.*, 2018). Salmonellae gets transmitted vertically among poultry, has longer persistence in the environment and poultry products are often contaminated with Salmonellae (Afshari *et al.*, 2018). Likewise, *L. monocytogenes* is a ubiquitous gram-positive bacterium widespread in the environment such as soil, water, animal gut, etc. *L. monocytogenes* grows over wide pH (4.39 to 9.40) range, refrigeration temperatures and is associated with listeriosis in human and animals (Singh *et al.*, 2012). Among 17 *Listeria* species, *L. monocytogenes* has been identified as the most significant member owing to its pathogenic nature to humans and animals responsible for food-borne infections, meningitis, encephalitis and febrile gastroenteritis (Barbuddhe *et al.*, 2002).

A rapid and sensitive method for detection of these pathogens will be absolutely useful for taking decision at field level about acceptance or the rejection of table egg lots. In the last decade, molecular techniques have appeared as most promising alternatives than conventional cultural methods in food microbiology. Conventional culture methods require series of procedure which consumes more time and are laborious. In such cases, use of molecular-based techniques thwart the delay and permits to detect the pathogens with greater sensitivity and reliability than conventional culture methods (Germini *et al.*, 2009; Rattanachaikunsopon and Phumkhachorn, 2012). A rapid and sensitive assay like polymerase chain reaction (PCR) based method for identification of the food borne pathogens is proven and even several pathogens can be detected in

a single reaction by multiplex PCR or mPCR (Soumet *et al.*, 1999).

Therefore, present study was designed with the objectives of simultaneous detection of *S. enterica* and *L. monocytogenes* using mPCR assay targeting *invA* and *prfA* genes respectively and comparison of mPCR with that of conventional method.

Materials and Methods

Sample collection: Table egg (360) were collected from different markets comprising of commercial layer farms (240) and backyard rearing (120) systems. Egg samples were collected from retail markets into sterile polybags and transported to the laboratory immediately. Samples were collected on four occasions at an interval of 15 days and analyzed for *S. enterica* and *L. monocytogenes* using the multiplex PCR.

Sample preparation: Pool of ten eggs selected from each batch were surface sterilized with 70% ethanol, broken under aseptic conditions and whole egg contents were mixed in bag for 3 minutes using stomacher (Bagmixer®, Interscience). Ten milliliters of resultant egg homogenates were then transferred into two separate sterile polybags containing 90 milliliters of buffered peptone water (BPW) or half Fraser Broth (hFB) for the primary enrichment (1:9) of *S. enterica* or *L. monocytogenes*, respectively. Bags containing BPW or hFB were mixed well in stomacher for 4 minutes and incubated at 37 °C and 30 °C for 24 hours for *Salmonella* and *Listeria*, respectively.

DNA isolation from primary enrichment: DNA was extracted from pre-enriched samples using snap chill method (Manoj *et al.*, 2014). Briefly, 2 mL pre-enriched broth (BPW or hFB) were centrifuged at 6,000 rpm for 10 minutes; resultant pellet containing bacterial cells was washed with sterile phosphate buffered saline and re-suspended in 50 µL nuclease free water. After boiling for 100 °C for 10 minutes, putative suspensions were subjected for one freeze-thaw cycle (-20 °C for 15 min.). Thawed suspension was centrifuged at 6,000 rpm for 10 minutes and supernatant collected into a fresh tube was used as template DNA for PCR.

Detection of *S. enterica* and *L. monocytogenes* in primary enrichment broth by PCR: For the detection of *S. enterica* and *L. monocytogenes* in the primary enrichment broth (BPW/hFB) multiplex PCR assay performed targeting

invA (invasion 'A' gene) of *S. enterica* (Rahn *et al.*, 1992) and *prfA* (transcriptional activator of virulence factor) of *L. monocytogenes* (Wernars *et al.*, 1992) as described by Germini *et al.*, (2009) with minor modifications.

Multiplex PCR was performed in 25 µL volume comprised of 12.5 µL of 2Xmaster mix (Himedia®), 0.5 µL of each primers [*Salmonella* genus specific *invA* gene primers 5'-AAT TAT CGC CAC GTT CGG GCA A-3' and 5'-TCG CAC CGT CAA AGG AAC C-3'; *L. monocytogenes* specific *prfA* primers 5'-TCA TCG ACG GCA ACC TCG G -3' and 5'-TGA GCA ACG TAT CCTCCA GAG T-3'], 3µL template DNA (1.5 µL each) and 7.5 µL of DNase free water. The mPCR amplification program consisted of initial denaturation for 5 min at 95°C, 40 cycles of denaturation (95°C, 60s), annealing (54°C, 40s) and elongation (72°C, 50s); followed by the final elongation at 72°C for 4 min. Amplified products were resolved in agarose gel (1.5%) and ethidium bromide stained amplicons were visualized under short-wavelength UV light source and photographed. Standard cultures of *S. Enteritidis* (ATCC 13076) and *L. monocytogenes* (MTCC 1143) were used for the standardization of mPCR.

Isolation and identification of *S. enterica* and *L. monocytogenes*: Samples showing positive by mPCR amplicons from the primary enrichment were subjected for conventional cultural isolation of *S. enterica* species and *L. monocytogenes* as per standard protocols (ISO 6579:2002 and ISO 11290:1998). Identification of *S. enterica* species and *L. monocytogenes* was carried out using a battery of biochemical tests (Blodgett, 2010).

Results and Discussion

Presence of *S. enterica* and *L. monocytogenes* was detected based on the amplification of the 284 and

217 base pairs PCR products in *Salmonella* and *Listeria* species, respectively (Figure 1). Among the table egg samples screened using the mPCR, *Salmonella* and *Listeria* were detected in 19.4 and 2.7 per cent, respectively. Distribution of these food safety hazards among different categories of table egg samples is given in Table 1. Occurrence of *S. enterica* was highest in backyard eggs followed commercial table eggs. Primary enrichment samples of commercial eggs were found negative for *L. monocytogenes* using *prfA* gene based mPCR. However, one sample of backyard eggs showed amplification for *prfA* gene.

Use of cultural media and methodology for detection and isolation of pathogens will vary and even sensitivity is also low and difficult to detect non-viable cells or injured cells of pathogens by cultural method, but they may have potential to recover and grow when the food is consumed, therefore molecular assay may be more appropriate. Several simplex or multiplex PCR assays have been developed for the rapid detection of *Salmonella* and *Listeria* species using specific primers (Germini *et al.*, 2009; Manning *et al.*, 2015; Liu *et al.*, 2015; Afshari *et al.*, 2018; Heymans *et al.*, 2018; Dzieciol *et al.*, 2016). Of the several amplification targets used for detection of *Salmonella* such as *agfA*, *fimA*, *viaB*, *fliC*-d, virulence-associated plasmids, etc the *invA* gene has been one of the most widely gene targets for the specific detection of *Salmonellae* (Phumkhachorn and Rattanachaikunsopon, 2017; Afshari *et al.*, 2018; Heymans *et al.*, 2018; Borges *et al.*, 2018). Likewise, *L. monocytogenes* could be specifically detected using virulence factors such as actin polymerization protein (*actA*), phosphatidylinositol phospholipase C (*plcA*), hemolysin (*hlyA*) and invasive associated protein (*iap*) genes that are involved in the pathogenesis (Liu *et al.*, 2015; Dzieciol *et al.*, 2016); of these, *prfA* has been the accepted target for the specific detection of *L.*

Table 1: Detection of *Salmonella enterica* and *Listeria monocytogenes* in eggs using mPCR

Egg source	Egg samples analyzed [#]	<i>S. enterica</i>	<i>L. monocytogenes</i>
Commercial layer farms	24	3 (12.5 %)	ND
Backyard	12	4 (33.3 %)	1 (8.3 %)
Total	36	7 (19.4 %)	1 (2.7 %)

[#]Each sample consists pool of 10 eggs; ND- Not detected.

Figure. 1: Multiplex PCR assay for screening of *S. enterica* (invA gene-278 bp) *L. monocytogenes* (prfA-217 bp)

M- 100 bp ladder, S-Standard (*S. Typhimurium* and *L. monocytogenes*), Lane 1 to 5 samples; Lane 6- NTC

monocytogenes (Wernars *et al.*, 1992).

The mPCR adopted in the present study was found to detect both pathogens simultaneously and the results of the mPCR were in complete concordance with the conventional cultural methods. However, separate primary enrichment broth for *Salmonella* and *Listeria monocytogenes* is preferred over universal primary enrichment medium while screening for pathogens in food samples using mPCR, where *Salmonella* will predominate over the *Listeria monocytogenes* multiplication irrespective of initial counts (Jofre *et al.*, 2005).

Similar studies by other investigators could detect few pathogen (Nagappa *et al.*, 2007; Gole *et al.*, 2013, Vinay *et al.*, 2016; Rajashekhara *et al.*, 2017) or have shown discrepancies (Al-Obaidi *et al.*, 2011; Safaei *et al.*, 2011; Paul *et al.*, 2016). The mPCR employed in the present study with minor modification was found reliable for the simultaneous detection of two major food safety hazards in table eggs with an anticipation of food safety compliance. Keeping in view, the limitations of laborious and time consuming conventional cultural methods, results of the present study support to propose application of mPCR is for the rapid and specific detection of *S. enterica* and *Listeria monocytogenes* in table eggs. Nevertheless, multi-centric validations involving large samples are warranted for the wide scale acceptance of results.

Conclusions

The applications of PCR have revolutionized the molecular diagnostics including detection of food safety hazards. In the present study, mPCR assay adopted for the rapid and simultaneous detection of *S. enterica* and *L. monocytogenes* in table egg contents. The results of the mPCR were in complete concordance with the conventional cultural methods. Therefore, the mPCR assay has been proposed as alternative tool for the rapid and simultaneous detection of *S. enterica* and *L. monocytogenes* in table eggs for the purpose of food safety compliance testing of samples.

Authorship contribution statement

Vinayananda C. O.: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft

Nadeem Fairoze: Conceptualization, Writing - review & editing, Visualization, Supervision, Funding acquisition.

C. B. Madhavaprasad: Conceptualization, Writing - original draft, Writing - review & editing.

Nagappa S. K: Methodology, Data curation, Writing - original draft, Writing - review & editing.

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Declaration of Competing Interest

All authors declare that there exist no commercial or financial relationships that could, in any way, lead to a potential conflict of interest.

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