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Culture-independent quantification of *Salmonellae* in food by molecular beacon based real-time PCR

Rajesh Singh Tomar, Anurag Jyoti*

Amity Institute of Biotechnology, Amity University Madhya Pradesh, Gwalior, India.

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ABSTRACT

The present study has explored the culture-independent quantification of *Salmonellae* in selected food items by targeting *invA* gene using molecular beacon (MB) based real-time PCR assay. The assay could detect ten Genome Equivalents/PCR (GE/PCR) of reference strain *S. typhimurium* MTCC 98. The assay could detect *Salmonellae* in selected food samples (vegetables, street foods, fruit juices) collected from Gwalior city. Coriander poses the highest concentration of *Salmonellae* ($1.058 \times 10^7 \pm 211734$ CFU/25g) in vegetables followed by panipuri ($6.73 \times 10^6 \pm 309502$ CFU/100 ml) in street food samples and Pineapple juice ($8.15 \times 10^3 \pm 293$ CFU/100 ml). Egg being the potential reservoir of *Salmonellae* was also contaminated ($5.2 \times 10^3 \pm 136$ CFU/25 g). The present assay is culture independent without pre-enrichment of samples. The quantification of *Salmonella* spp. in food samples can be completed in 4-6 h by the assay developed in the present study, in comparison to culture based method which requires 2 to 3 days.

INTRODUCTION

Salmonella, prevalent in food is one of the deadliest pathogens, causes major health and economic losses worldwide (Agarwal *et al.,* 2015; de Jong *et al.,* 2006; Voetsch, *et al.,* 2004). Eggs, poultry meat, fruits and vegetables are the important sources of human salmonellosis (Hald *et al.,* 2004). In a developing country like India, infections due to contaminated food are oftenly reported. India has been categorized under the high risk zone of typhoid caused by *Salmonella* serovars. Most of the disease incidences occur due to the improper management and lack of quantitative data. Identification of potentially pathogenic *Salmonella* serovars is currently a labor intensive process. The initial isolation of this microbe from food samples is difficult as the small numbers of bacteria can be present in the sample (Baylis *et al.,* 2000). This requires the preenrichment step prior the quantification. The current established method for isolation of *Salmonella* from food matrices is a well established procedure–ISO 6579, which is laborious and time-consuming and takes almost 5 days to complete (Tomar *et al.,* 2015; Agarwal *et al.,* 2014; Uyttendaele *et al.,* 2003). Kauff-man-White serotyping system is used to characterize *Salmonella* into its subspecies, based on the variability of the O, H and Vi antigens (Yoshida *et al.,* 2007). The serotyping method is unable to identify *S. enterica* which lack either the O antigen alone or both the O and the H antigens (Hoorfar *et al.,* 2000). Polymerase Chain Reaction (PCR) is the modern nucleic acid based diagnostic method has been explored for the detection of *Salmonella* serovars in food samples (Malkawi *et al.,* 2003).

Though the PCR is specific technique, it still lacks the sensitivity and a post PCR processing is required, which limit its establishment for diagnosis of *Salmonella*. The *invA* gene encodes invasion associated protein is present in *Salmonella* serotypes including all subspecies (Galan *et al.,* 1996; Malorny *et al.,* 2003; Hadjinicolaou *et al.,* 2009). The *invA*, being the signature gene is used for quantification of *Salmonella* spp. in food samples.

* *Corresponding Author*
Dr. Anurag Jyoti, Amity Institute of Biotechnology,
Amity University Madhya Pradesh, Gwalior, India.
Email ID: ajyoti @ gwa.amity.edu, Phone: +91-7898805402

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Therefore, there is an urgent need for the development of assay for the specific and sensitive detection of *Salmonella* in food samples. Quantitative PCR, oftenly called as real-time PCR is a major revolution in molecular diagnostic methodologies. Real-time PCR is highly sensitive, specific and allows accurate quantification of the target DNA. Real-time PCR through use of fluorescence detection strategies allows the quantification of nucleic acid in a reaction without post-PCR processing. Real-time PCR based methods with advantages of quantification, sensitivity, and rapidity, have been reported to detect *Salmonella* in various food items (Liming *et al.,* 2004; Bhagwat, 2004; Park *et al.,* 2008; Singh *et al.,* 2011). Furthermore, a number of commercial real-time PCR systems have been used for detection of *Salmonella* in meat samples (Perelle *et al.,* 2004; Cheung *et al.,* 2004; Patel *et al.,* 2006). However, majority of these assays need pre-enrichment of the samples and often require 17-20 h for quantitative enumeration of pathogen.

The present study aims to evaluate the culture-free highly sensitive quantitative PCR assay using MB probe for quantification of *Salmonellae* in various food matrices collected from local market in Gwalior city.

MATERIALS AND METHODS

Primers and probe

In the present study, the *invA* primer set (5´-GTGAAATTATCGCCACGTTCGGGCAA-3’ and 5´-TCATCGCACCGTCAAAGGAACC-3’), was adopted (Rahn *et al.,* 1992). The primer set, producing 284 bp shows high specificity for all subspecies of *Salmonella* serotypes (Malorny *et al.,* 2003). To ascertain the *in-silico* specificity, the primers wereanalysed by nucleotide BLAST programme.

The molecular beacon for specific detection of *Salmonella* spp. harboring *invA* gene was adopted from Jyoti et al. (2010). The molecular beacon (5’-<u>CCAGGC</u>TTCCAGTACGCTTCGCCGTTC<u>GCCTGG</u>-3’) was analysed for correct stem loop configuration secondary structure formation in mFold server (http://mfold.rna.albany.edu/?q=mfold/DNA-Folding-Form) to ensure correct folding of the probe. The MB was modified at 5’ end with fluorophore (FAM: 6- carboxy–fluorescein) and at 3’ end with quencher DABCYL.

Bacterial strains and specificity of the assay

Bacterial strains used to determine the specificity of PCR primers are listed in table 1. The reference strains (*S. typhimurium* MTCC 98, *S. typhi* MTCC 735) were procured from Microbial Type Culture Collection (MTCC) at Institute of Microbial Technology (IMTECH), Chandigarh, India.

S. typhimurium MTCC98 was used for generation of standard curve for the real-time PCR. The inclusivity and exclusivity of the computed MB probe was checked using reference strains of *Salmonella* and other genera.

Food samples

Street food items included Panipuri (water), Egg, Noodles and fruit juices like Citrus (*Citrus limetta*), Pineapple (*Ananas comosus*), Sugarcane (*Saccharum* spp.) and Watermelon (*Citrullus lanatus*) were procured from local markets in Gwalior city. Apart from these vegetables used for garnishing the dishes such as fenugreek (*Trigonella foenum-graecum*), mint (*Mentha* spp.) and coriander (*Coriandrum sativum*) leaves were purchased from local markets. Fruit juices and water of panipuri (2 litres each) were filtered through sterilized muslin clothes to remove the coarse fibres. The leafy vegetables, 50 g each) were properly rinsed in 200 ml saline with gentle shaking followed by sonication (cycle 0.5 for 20 s) with output power of 200 W to transfer the microflora in saline. The sample volume was then reduced to 500 µl by centrifugation at 14000 x g.

Isolation of multigenomic DNA

Multigenomic DNA was prepared using boiling prep method (Jyoti *et al.,* 2010). Briefly, the concentrated sample was heated to boiling temperature for 30 minutes to ensure killing of all microflora followed by the release of their nucleic acids. The DNA was removed by centrifuging the debris at 8,000 x g for 5 min. Further, the DNA in supernatant was precipitated using sodium acetate (0.3 M, pH 5.2) and ice cold ethanol. The precipitated DNA was pelleted by centrifugation at 12000 x g for 5 min. DNA pellet was resuspended in 100 µl TE, pH 8 after washing thrice with 70% ethanol. The purity and yield of isolated DNA was determined using Spectrophotometer.

Quantitative enumeration of *Salmonellae* using real-time PCR

The virulent *invA* gene of *Salmonella* was targeted to quantify the *Salmonellae* in real life samples by real-time PCR. To determine the limit of detection of molecular beacon based quantitative PCR assay, a series of 10-fold serially diluted *Salmonella typhimurium* MTCC 98 pure culture genomic DNA (10^7–1 GE/PCR) was analysed for real-time PCR amplification. Template DNA, primers and probe were added to PCR master mix. The final concentration of MB probe and primers were 200 nM and 400 nM respectively. The temperature conditions were set as follows: initial denaturation of 95 °C for 12 min, followed by 48 cycles at 94 °C for 30 s, 54 °C for 30 s and 72 °C for 30 s. Similar assay was performed with the *Salmonella typhi* MTCC 733, another strain of *Salmonella*. Purified multigenomic DNA (5 µl) from environmental samples were diagnosed for under identical PCR conditions. This resulted in generation of a standard curve to quantify the load of *Salmonellae* in different food samples.

RESULTS AND DISCUSSION

Generation of standard curve

The present molecular beacon based assay detected 10 or more genomic equivalent (GE) of the reference strain per PCR per PCR of *S. typhimurium* MTCC 98 (Fig. 1).

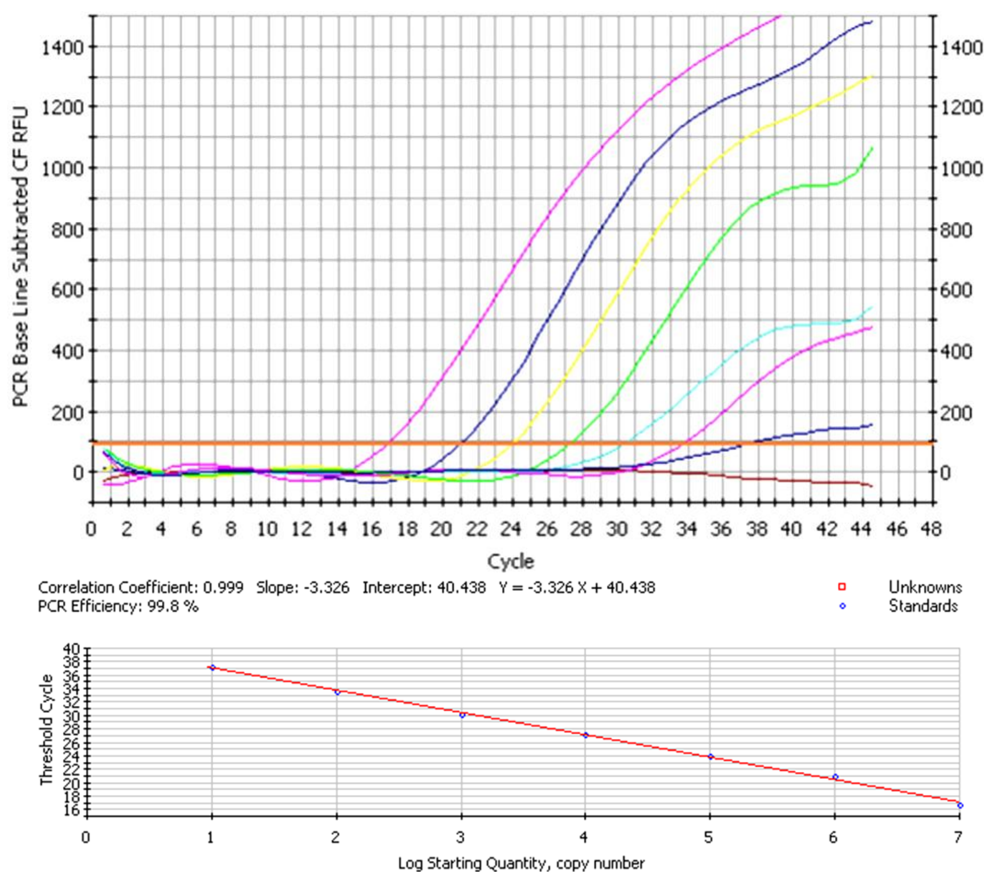

Fig. 1: Real-time PCR spectra (A) and Standard curve (B) obtained from 10^7 down to 10 GE/PCR genomic DNA of *S. typhimurium* MTCC 98.

Specificity of the test

The developed real-time PCR assay is highly sensitive and specific to the *Salmonella* spp. The reference strains of *Salmonella* were positive for the *invA* gene. There was no amplification observed in *E. coli* reference strains. Recovery of the target organism or its nucleic acids from sample matrix is important for its detection. Reference strains of *Salmonella* were detected by the assay, whereas other genera were found negative. The assay developed based on *invA* gene is highly specific and sensitive for detection of *Salmonella* even in street food samples.

Table 1: Specificity of the molecular beacon targeting *invA* gene for detection of *Salmonella* spp

Reference Strains	*invA* gene
Salmonella typhimurium MTCC 98	+
Salmonella typhi MTCC 733	+
E. coli MTCC723 (MTCC, Chandigarh)	-

Detection of *Salmonellae* in food samples

Food samples collected from Gwalior city were analysed for the presence of *Salmonella* spp. Panipuri (water), Egg, Noodles and fruit juices like Citrus, Pineapple were significantly contaminated with *Salmonellae*. Sugarcane and Watermelon juices were negative for the presence of *Salmonellae*. Leafy vegetables were contaminated with *Salmonellae* which on consumption may pose health risk to consumers. The contamination levels of *Salmonellae* were varied significantly. Coriander poses the highest concentration of *Salmonellae* ($1.058x10^7$±211734 CFU/25g) in vegetables followed by panipuri ($6.73x10^6$±309502 CFU/100 ml) (Table 2) in street food samples and Pineapple juice ($8.15x10^3$±293 CFU/100 ml) (Table 3). *Salmonellae* are the leading food borne pathogens and can enter the food chain at any level (Malorny *et al.,* 2008). A variety of food matrices are the potential reservoirs of *Salmonella*. Presence and long term persistence of *Salmonella* in surface waters is well documented (Moganedi *et al.,* 2007; Jyoti *et al.,* 2010). Leafy vegetables grown near the rural and urban interface of city are irrigated through the contaminated river/ pond water. Apart from these, the vegetables on the river bank are in constant exposure of this microbial flora. These contaminated vegetables are transported to the local markets and are improperly washed. In the present study, all the leafy vegetables which are used to garnish the dishes were found contaminated with S*almonellae*.

Egg, being the potential reservoir of *Salmonellae* was also contaminated ($5.2x10^3$±136 CFU/25 g). Poultry samples are the primary reservoirs of *Salmonellae*. Egg shells bearing fecal matter of chickens are contaminated with the *Salmonella* are stored at room temperature, which helps these organisms to multiply fast during transportation and handling (Jamshidi *et al.,* 2010). Sugarcane and Watermelon were negative for the presence of *Salmonellae* (Table 3).

Table 2: Contamination of vegetables used for garnishing the dishes and street food items by *Salmonellae*.

S. No.	Food samples	*Salmonellae* (CFU/25 g produce)
Vegetables used for garneshing the dishes		
1.	Fenugreek (Methi)	$1.57x10^6 \pm 72220$
2.	Mint	$1.02x10^6 \pm 35700$
3.	Coriander	$1.058x10^7 \pm 211734$
Street foods		
1.	Panipuri	$6.73x10^6 \pm 309502$
2.	Egg	$5.2x10^3 \pm 136$
3.	Noodles (Chowmin)	$9.1x10^2 \pm 22$

*Values are mean (n = 5) ± S.D.

Table 3: Contamination of street fruit juices by *Salmonellae*.

S. No.	Juices	*Salmonellae* (CFU/ml)
1.	Citrus (Mausambi)	$3.4x10^3 \pm 79$
2.	Pineapple	$8.15x10^3 \pm 293$
3.	Sugarcane	N.D.
4.	Watermelon	N.D.

*Values are mean (n = 5) ± S.D. N.D. Not detected.

The rapidity and sensitivity of detection technique plays an enormous impact on quantitative microbial risk assessment for generation of quantitative data on food samples. A number of techniques are available which rely on the enrichment of sample prior to PCR (Myint *et al.,* 2006; Malorny *et al.,* 2007). In a study, an 18 h of pre-enrichment was required for detection of all five chicken samples (Myint *et al.,* 2006).

The present culture independent assay is more rapid than other previous reports (Singh *et al.,* 2011; Hadjinicolaou *et al.,* 2009; Malorny *et al.,* 2007; Bohaychuk *et al.,* 2007) which require at least 6, 8, 24 and 52 h for detection of *Salmonella* in food and other samples. The present assay is culture independent without pre-enrichment of samples. The quantification of *Salmonella* spp. in food samples can be completed in 4-6 h by the assay developed in the present study, in comparison to culture based method which requires 2 to 3 days. Therefore, the culture-independent real-time PCR assay developed in this study for quantitative enumeration of *Salmonella* spp. in food samples is rapid, accurate and sensitive.

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