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Multiplex-PCR assay for detection of some major virulence genes of *Salmonella enterica* serovars from diverse sources

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The virulence of *Salmonella* depends on virulence factors encoded by specific genes. The present study describes the development of a simple and rapid multiplex PCR (m-PCR) assay for simultaneous detection of seven major virulence genes of *Salmonella* (*inv*A, *inv*H, *stn*, *sop*B, *sop*E, *sef*C and *pef*A). Presence of these genes was studied using 17 standard cultures and 76 field isolates from different sources. Seventeen non-*Salmonella* strains were also tested for specificity of the optimized PCR. A spiked control experiment was done to detect the sensitivity of m-PCR assay. The primer pairs were found to be specific for *Salmonella* only. The assay detected 250 pg of purified chromosomal DNA, or 12 cfu of *Salmonella* in crude lysates. All the field isolates and standard strains of *Salmonella* were found to carry *inv*A, *inv*H, *stn* and *sop*B genes, while variability was observed with respect to *sop*E, *sef* C and *pef*A genes. Thus this multiplex PCR assay provides a simple and rapid method for detection of major virulence factors in important clinical serovars of *Salmonella*.

Keywords: Multiplex PCR, *Salmonella*, spiking, virulence genes.

SALMONELLA, the Gram-negative bacillus of family Enterobacteriaceae, is widely distributed in nature and can cause diseases ranging from gastroenteritis to typhoid fever. More than 2500 serovars of *Salmonella* are known so far. *Salmonella enterica* subsp. *enterica* is one of the leading causes of zoonotic food-borne disease worldwide[1]. *Salmonella* infections exhibit a complex pathogenesis in which numerous virulence genes are involved[2]. These genes are clustered within *Salmonella* pathogenicity

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islands (SPIs) and 21 such islands (SPI-1 to SPI-21) have been reported to date[3]. However, different serovars show variations in occurrence of these SPIs and individual genes[4]. Some genes such as *inv*, *sef*, etc. are involved in adhesion and invasion[5,6], while others are like *mgtC5* associated with survival in the host system, or in the actual manifestation of pathogenic processes, viz. *sop*, *stn*, *pip A, B, D*, etc.[7–9]. Serovars like *S.* Typhimurium also harbour self-transmissible virulence plasmids (pSLTs) carrying virulence genes such as the *spv* operon, involved in intramacrophage survival, and the plasmid-encoded fimbriae (*pef*) fimbrial operon[10,11]. PCR assays have been developed for the detection of different gene-encoded virulence factors, viz. simplex PCR for *Salmonella* enterotoxin (*stn*)[12], *Salmonella enteritidis fimbriae* (*sef*) and *plasmid encoded fimbriae* (*pef*)[13], and multiplex PCR (m-PCR) assays for a combination of a few such virulence factors[14–16]. Here, we report the development of a m-PCR for rapid and simultaneous detection of seven major virulence genes of *S. enterica*.

Seventeen standard cultures of *Salmonella* obtained from Microbial Type Culture Collection (MTCC), Chandigarh and National Institute of Cholera and Enteric Diseases (NICED), Kolkata, India were used for determination of sensitivity and specificity of the PCR assay (Table 1). A total of 17 non-*Salmonella* bacterial isolates mentioned elsewhere were used to check the specificity of PCR assay. Besides, 76 other *Salmonella* isolates obtained from faecal samples of different species of animals and birds (domestic and wild), including humans (children and adults with diarrhoea) were used to screen the virulence genes using PCR assay (Table 2).

Specific primers were designed for conserved regions of the gene sequences using on-line primer BLAST software of NCBI. Table 3 shows the GenBank accession numbers of the gene sequences used. Sequences of all the primers were evaluated using Primer-BLAST and BLASTn tools (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to identify any non-specific targets and ensure specificity of the PCR assay. The PCR products ranged from 185 to 942 bp in length. Proper care was taken to make sure that the primer pairs had similar annealing temperature and the resulting amplicons had a minimum 50 bp difference among them. The primers were synthesized by Integrated DNA Technologies (IDT), USA.

For standardization of m-PCR protocol, the bacterial DNA was isolated by a bacterial DNA isolation kit (Mo-Bio, USA), according to the instructions of the manufacturer. The purity and quantity of the extracted DNA were checked by Nanodrop spectrophotometer (ND 1000, Thermo Scientific, USA). Boiling lysis method of DNA extraction was followed for detection of various genes in different isolates of *Salmonella*[17]. DNA from the non-*Salmonella* isolates was also extracted by the same method. Briefly, all the bacterial cultures other than *Clostridium* species were grown aerobically in Luria Bertani (LB) broth overnight at 37°C, while the clostridial cultures were grown in brain heart infusion (BHI) broth at 37°C for 24–48 h in an anaerobic jar. Next, 1 ml each of the above cultures was pelleted down by centrifugation at 15,322 *g* (relative centrifugal force) for 5 min. The pellet was resuspended in 100 µl of 1× Tris EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and transferred to a micro-centrifuge tube. Bacterial suspension was boiled in a water-bath for 10 min and then immediately kept in ice for 10 min. It was again centrifuged at 15,322 *g* (rcf) for 10 min and the supernatant containing genomic DNA was collected. Quantification and purity of the freshly extracted DNA were checked with the help of Nanodrop-1000 Spectrophotometer and used immediately in the PCR reaction.

Initially, presence of virulence genes in the standard strains was confirmed by performing simplex PCR. m-PCR was optimized before the final experiment. Parameters optimized for m-PCR included primer concentration (0.2–0.5 µM), $MgCl_2$ concentration (2–3.5 mM), annealing temperature (50–60°C), annealing time (30–90 sec) and extension time (30–120 sec). Initially, each of the genes was amplified by simplex PCR using the same reaction conditions, i.e. 2 mM $MgCl_2$, 0.2 µM primer concentration for all genes, 50°C annealing temperature, 30 sec annealing time and 30 sec extension time. However, when the same conditions were used in m-PCR, no amplification was observed. Gradually all the parameters were adjusted step by step by applying them in different combinations. While amplification was observed for *pefA* and *sefC* genes with the combinations of 3.5 mM $MgCl_2$, 0.2 µM primer concentration for all primers, 56°C annealing temperature, 90 sec annealing time and 120 sec extension time, primer concentration for the remaining five genes (*invA*, *invH*, *stn*, *sopB* and *sopE*) had to be increased up to 0.4 µM for optimizing amplification (Table 1). Finally m-PCR parameters were established with 3.5 mM $MgCl_2$ concentration and a PCR cycle of initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 30 sec, annealing at 56°C for 90 sec, extension at 72°C for 2 min and final extension at 72°C for 10 min. The m-PCR was carried out with a total reaction volume of 25 µl. The reaction mixture contained 12.5 µl of 2× PCR master-mix (Dream *Taq* PCR Master-Mix, Fermentas, USA), 1.5 µl of $MgCl_2$ (25 mM), 2.0 µl (40–60 ng) of template DNA and the final volume was made up with nuclease-free water. PCR amplification was performed in S1000 Thermal Cycler (Bio-Rad, USA). An aliquot (10 µl) from the PCR products of each sample was analysed by gel electrophoresis in 2% agarose gel containing ethidium bromide (0.5 µg ml^{-1}). The gel was visualized under a gel documentation system (GelDoc XR + system, Bio-Rad) and images were captured by Image Lab Software. Control reaction mixtures containing no template and negative control were also included in each of the sample runs.

Table 1. Virulence gene profiles of standard strains of *Salmonella*

Isolates	Strain no.	Virulence genes						
		invA	*invH*	*stn*	*sopB*	*sopE*	*sefC*	*pefA*
S. Newport	MTCC 3229	+	+	+	+	+	–	–
S. Schwarzengrund	MTCC 3230	+	+	+	+	–	–	–
S. Typhimurium	MTCC 98	+	+	+	+	+	–	+
S. Typhimurium	MTCC 3917	+	+	+	+	–	–	+
S. Virchow	MTCC 1166	+	+	+	+	+	–	–
S. Abony	NCTC 6017	+	+	+	+	+	–	–
S. Choleraesius	ATCC 10708	+	+	+	+	+	+	–
S. Enteritidis	IDH 5073	+	+	+	+	+	+	–
S. Idikan	NICED 503984	+	+	+	+	+	+	+
S. Infantis	NICED 505330	+	+	+	+	+	–	–
S. Paratyphi A	NICED C6915	+	+	+	+	+	–	–
S. Paratyphi B	NICED NK3727	+	+	+	+	+	–	–
S. Poona	NCTC 4840	+	+	+	+	+	–	–
S. Typhi	NICED C6953	+	+	+	+	+	+	–
S. Vellore	ATCC 15611	+	+	+	+	+	–	–
S. Virdi	IDH 377	+	+	+	+	+	–	–
S. Worthington	IDH 3642	+	+	+	+	+	–	–

Table 2. Virulence gene profiles of field isolates of *Salmonella*

Isolates	Source	No. of isolates	Virulence genes						
			invA	*invH*	*stn*	*sopB*	*sopE*	*sefC*	*pefA*
S. Dublin	Cattle	5	+	+	+	+	–	–	–
S. Enteritidis	Poultry	6	+	+	+	+	+	+	+
S. Enteritidis	Wild bird	6	+	+	+	+	+	+	+
S. Enteritidis	Human	3	+	+	+	+	+	+	+
S. Gallinarum	Wild bird	2	+	+	+	+	+	–	–
S. Gallinarum	Poultry	2	+	+	+	+	+	–	–
S. Kentucky	Tiger	2	+	+	+	+	–	–	–
S. Litchfield	Poultry	2	+	+	+	+	+	–	–
S. Newport	Poultry	3	+	+	+	+	+	–	–
S. Newport	Cattle	2	+	+	+	+	+	–	–
S. Newport	Human	2	+	+	+	+	–	–	–
S. Typhi	Human	2	+	+	+	+	–	+	–
S. Weltevreden	Poultry	7	+	+	+	+	–	–	–
S. Weltevreden	Wild bird	7	+	+	+	+	–	–	–
S. Weltevreden	Pig	7	+	+	+	+	–	–	–
S. Weltevreden	Kako snake	2	+	+	+	+	–	–	–
S. Choleraesius	Pig	6	+	+	+	+	+	+	–
S. Typhimurium	Pig	7	+	+	+	+	+	–	+
S. Typhimurium	Human	3	+	+	+	+	+	–	+

Sensitivity of the m-PCR assay was tested using *S. enterica* serovar enteritidis (IDH 5073) and Typhimurium (MTCC 98) strains to evaluate the minimal quantity of genomic DNA as well as cells required for their detection. To determine sensitivity, purified genomic DNA extracted using DNA isolation kit was serially diluted tenfold with sterile double-distilled water and amplified by PCR. To determine minimal cells required, overnight cultures of both strains were serially diluted tenfold up to 10^{-10} and viable counts were determined by plating 100 µl of each dilution of the bacterial culture on Brilliant Green Agar (BGA) plates and then incubated at 37°C for 18 h. The counts were expressed as cfu ml^{-1}. Finally, genomic DNA was isolated from 1 ml of the diluted cultures and amplified again. The number of cells/reaction of m-PCR or number of cells ml^{-1} of culture required for detection by m-PCR was counted from the amount of template (2.0 µl) added in the m-PCR reaction. A panel of 17 non-*Salmonella* strains, viz. *Bacillus subtilis*, *Clostridium difficile*, *Clostridium perfringens*, *Escherichia coli* ($n = 6$), *Klebsiella pneumoniae*, *Pasteurella multocida*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Shigella dysentery*, *Staphylococcus aureus*, *Streptococcus mutans* and *Pneumococcus pneumoniae*,

Table 3. Details of the primers used in the present study

Target gene	Primer sequence (5′–3′)	Primer concentration (μM)	Product size (bp)	Accession no.
invA	FP-ACCACGCTCTTTCGTCTGG RP-GAACTGACTACGTAGACGCTC	0.2	942	AE006468.1
invH	FP-TATAGCTGTCTTCCTGTCTT RP-ATGTATTGTGGATGTTCCTG	0.2	305	AE006468.1
stn	FP-ATTGAGCGCTTTAATCTCCT RP-GCTGTTGAATCTGTACCTGA	0.2	543	AE006468.1
sopB	FP-AGCATCTCTAAACGCTACTG RP-GCTTCTATCACTCAGCTTCA	0.2	470	AE006468.1
sopE	FP-GGTAGGGCAGTATTAACCAG RP-TTTATCTCCCTAGGTAGCCC	0.2	254	AE014613.1
pefA	FP-GCCAAAGTACTGGTTGAAAG RP-TATTTGTAAGCCACTGCGAA	0.4	185	AE006471.1
sefC	FP-GGCAGGTCCAAAACTATACA RP-GCGATAACGAAACACCATTT	0.4	609	AE014613.1

FP, Forward primer; RP, Reverse primer.

were tested for specificity using the optimized m-PCR conditions as mentioned above. The procedure was repeated twice to ensure reproducibility.

Spiking of stool sample was performed according to Pathmanathan *et al.*[18] with some modifications. Briefly, 1 g of stool sample from a healthy individual was mixed with 10 ml of BHI broth. Then a known number of *Salmonella* Typhimurium cells was mixed with the faecal suspension to obtain a final concentration of 1, 10, 100, 1000 and 10,000 cfu ml^{-1} and incubated for 10 h. The inoculated samples were harvested after 0, 5 and 10 h and PCR was performed using extracted DNA from the cultures. The sensitivity of PCR is defined as the lowest concentration of *Salmonella* Typhimurium (in cfu ml^{-1} or cfu PCR^{-1}) that gives positive results. Faecal suspension without spiking was included in every PCR run as negative control to rule out false positives.

Two hundred and ninety-five diarrhoeic faecal samples/intestinal contents from poultry (n = 52), pig (n = 60) and humans (n = 43), faecal samples of domestic (cattle = 60), zoo animals (*Gekko gecko* = 10, *Panthera tigris* = 10) and wild birds (n = 60) were collected from various sources. All the faecal samples/intestinal contents were examined for the presence of *Salmonella* following standard enrichment and isolation procedures and also by m-PCR method. For this, 1 g of sample was inoculated in 10 ml of buffered peptone water and incubated at 37°C for 24 h. About 1 ml of pre-enriched culture was transferred to 9 ml of tetrathionate broth (Himedia, India) and incubated at 37°C for 24 h. Subsequently, a loopful of broth was streaked on the surface of BGA and xylose lysine deoxycholate (XLD) agar (Himedia) and incubated at 37°C for 24 h. Suspected colonies were further purified on MacConkey's lactose agar and biochemical tests were carried out by conventional methods[19]. The isolates were also serotyped at NICED, Kolkata. Genomic DNA (2 μl) prepared from 1 ml enrichment broth (5 h in buffered peptone water) by simple boiling method was used in m-PCR. The isolates were tested further by m-PCR for different virulent genes.

S. enterica subspecies *enterica* was detected in 76 (28.75%) out of 295 collected samples and ten serotypes were isolated, namely *S.* Typhi, *S.* Typhimurium, *S.* Newport, *S.* Dublin, *S.* Enteritidis, *S.* Gallinarum, *S.* Sitchfield, *S.* Weltevreden, *S.* Choleraesuis and *S.* Kentucky. In humans 10 (23.25%) out of 43, in pig 20 (33.33%) out of 60, in cattle 7 (11.66%) out of 60, in poultry 20 (38.46%) out of 52, in wild bird 15 (25%) out of 60, in tiger 2 (20%) out of 10, and in gecko 2 (20%) out of 10 samples showed the presence of *S. enterica*. All the standard strains as well as 76 field isolates of *Salmonella* were found to be positive for *inv*A, *inv*H, *stn* and *sop*B genes, while there was variable presence of *sop*E (55.27%), *sef*C (30.26%) and *pef*A (32.90%) genes in the field isolates and 88.24%, 23.53% and 17.65% respectively, in the standard strains (Tables 2 and 3). All the *Salmonella* isolates yielded 942, 305, 470 and 543 bp amplicons for *inv*A, *inv*H, *sop*B and *stn* genes respectively, while the amplicon sizes for *sop*E, *sef*C and *pef*A were 254, 609 and 85 bp respectively (Figure 1). Overall, the results indicate high specificity of m-PCR, as it detected the presence of virulence genes in all the standard strains and field isolates of *Salmonella.* The m-PCR assay could detect approximately 250 pg of purified chromosomal DNA, or 12 cfu (5.9×10^3 cfu ml^{-1}) *Salmonella* cells in crude lysate. In the spiked control experiment, m-PCR could detect *Salmonella* Typhimurium at 0 h in the spiked sample containing 10^4 cfu ml^{-1} (20 cfu PCR^{-1} reaction in

2 µl template). However, all the spiked samples containing 10 and 1000 cfu ml^{-1} were found to be positive by m-PCR after 5 and 10 h of pre-enrichment. Three out of the five spiked samples containing one cfu ml^{-1} were found to be positive after 5 and 10 h of incubation. No amplicons of corresponding sizes were detected in case of non-*Salmonella* strains, indicating the specificity of the assay. Repeated PCR amplifications gave similar reproducible results.

The *inv*A gene of *Salmonella* contains sequences unique to this genus and has been proved to be a suitable PCR target with potential diagnostic application[18]. This gene is recognized as an international standard for detection of *Salmonella* genus[19]. The *inv*H gene was detected in all the *Salmonella* isolates irrespective of serovars by PCR[20]. However, it was not detected in many of the isolates tested by microarray analysis. The *stn* gene has been proposed as a putative virulence factor and causative agent of diarrhoea[6]. Detection of *stn* gene was reported in all the strains of *S. enterica*, but not in *S.* Bongori[7].

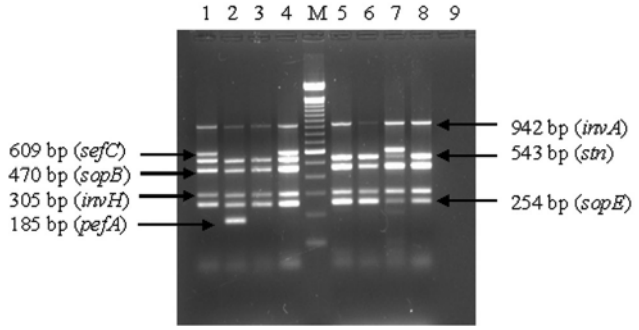

Figure 1. Detection of virulence genes of *Salmonella* by multiplex-PCR (m-PCR). Lane M, 100 bp ladder; lane 1, *S.* Typhi; lane 2, *S.* Typhimurium; lane 3, *S.* Virchow; Lane 4, *S.* Choleraesuis; lane 5, *S.* Paratyphi A; lane 6, *S.* Paratyphi B; lane 7, *S.* Enteritidis; lane 8, *S.* Newport; lane 9, Negative control.

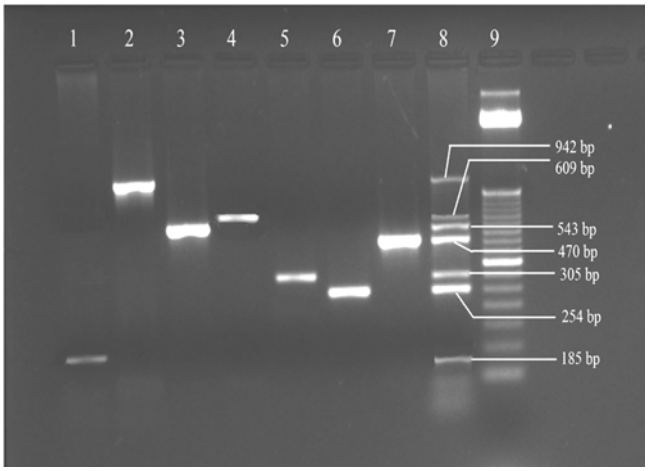

Figure 2. Detection of seven genes in simplex PCR and m-PCR. Lane 1, *pefA* (185 bp); lane 2, *invA* (942 bp); lane 3, *stn* (543 bp); lane 4, *sefC* (609 bp); lane 5, *invH* (305 bp); lane 6, *sopE* (254 bp); lane 7, *sopB* (470 bp); lane 8, m-PCR of *S.* Idikan; lane 9, 50 bp ladder (Invitrogen).

Salmonella outer proteins (Sop) are the effector molecules of type-III secretion system (TTSS) which are involved in the early stage of *Salmonella* infection. These proteins are encoded by *sop* genes and several polymorphisms of these genes have been identified (*sop*A–*sop*E)[8]. Among the different types of Sop proteins (SopB, a 60 kDa protein), initially identified in the culture filtrate of *S. enterica* serovar Dublin, was found to be associated with *Salmonella*-induced diarrhoea and gastroenteritis[7]. We detected *sop*B gene in all the serovars of *Salmonella* tested, irrespective of host species and place of origin. Our finding is similar to that of Rahman[22], who detected *sop*B gene by PCR in all tested serovars of *Salmonella* from humans and animals. On the contrary, the *sop*E gene was found to be present in 15 of the 17 standard *Salmonella* strains and was absent in *S.* Schwarzengrund and *S.* Typhimurium. Similarly, it was detected in the field isolates of *S.* Enteritidis, *S.* Gallinarum, *S.* Litchfield and *S.* Newport. However, variation was observed in the same serovars (e.g. *S.* Newport and *S.* Typhimurium). Our findings are similar to those of Rahman *et al.*[23,24], who also detected *sop*E gene in *S.* Enteritidis, *S.* Gallinarum and *S.* Virchow isolates by PCR. The product of this gene is shown to play a role in *Salmonella* virulence; however, its absence from multiple invasive *Salmonella* isolates suggests that it is unnecessary for invasive manifestation in humans[25]. In the present study, presence of *pef*A gene was also evaluated, which encodes for the major portion of the *pef* operon. The *pef*A gene was detected in 3 of the 17 standard *Salmonella* strains, and 25 of the 76 *Salmonella* isolates belonging to serovars *S.* Enteritidis, *S.* Typhimurium and *S.* Idikan among field isolates. Our findings are in agreement with those of Murugkar *et al.*[26], who detected the *pef* gene in *S.* Enteritidis, *S.* Paratyphi B and *S.* Typhymurium isolates from humans, animals and birds. They found it to be absent in *S.* Bareily isolates. However, they recorded variation among isolates of the same serovar. We also could not detect this gene in the standard strains of *S.* Enteritidis and *S.* Paratyphi *B* tested. This inter-serovar variation in the presence of *pef* gene has also been recorded previously[13]. However, few researchers could not detect *pef* gene in *S.* Typhi, *S.* Paratyphi A and *S.* Typhimurium[27]. *Salmonella* Enteritidis fimbriae 14 (SEF 14) is one of the major fimbriae of *Salmonella*, encoded by fimbrial operons *sef*A, *sef* B, *sef* C and *sef* D[5]. *SefC* forms the largest component of the fimbriae and its presence indicates SEF adhesion. The *sef* C gene was found to be present only in *S.* Enteritidis, *S.* Typhi, *S.* Choleraesius and *S.* Idikan serovars of both field isolates and standard strains of *Salmonella*. The present findings are more or less similar to those of Rahman *et al.*[13], who showed that except for strains of *S.* Enteritidis and *S.* Gallinarum, none of the other serovars, viz. *S.* Typhimurium, S. Newport, *S.* Kentucky, *S.* Weltevreden

and *S*. Indiana harboured this gene. Similarly, researchers also recorded this gene in field isolates of *S.* Enteritidis of human and animal origin only[26]. In our study, *sef*C gene was found to be absent in *S*. Typhimurium, *S*. Newport, *S*. Kentucky, *S*. Weltevreden, *S*. Choleraesius, *S*. Virchow, *S*. Schwarzengrund, *S*. Paratyphi B, *S*. Infantis, *S*. Abony, *S*. Poona, *S*. Vellore, *S*. Virdi and *S*. Worthington. The *sef*A gene was detected in *S*. Dublin, in addition to *S*. Enteritidis[27].

The reaction condition for m-PCR assay was optimized to ensure that all the target virulence gene sequences were satisfactorily amplified. The annealing temperature of 56°C was selected because at this temperature adequate resolution of all the amplified products could be seen without any non-specific bands. The primer concentration was optimized after standardizing individual simplex PCRs for the studied genes and showed similar annealing temperatures without any non-specific amplification. m-PCR also confirmed the virulence gene profile (Table 3) of the isolates without any non-specific bands (Figure 2). m-PCR had a reasonably high level of sensitivity in spiked human stool samples. m-PCR in the present study had a reasonably high level of sensitivity and was able to detect as low as 10 organisms per gram of stool sample following 5 h enrichment in buffered peptone water. Lim *et al.*[28] reported that the sensitivity of m-PCR was 500 ng DNA of *S.* Enterica serovar Typhimurium. Kapley *et al.*[29] reported higher sensitivity of the PCR reaction with 10^2 cells ml^{-1} for amplification of *inv*A, *lam*B and *ctx*A genes of *Salmonella* Typhi, *E. coli* and *Vibrio cholerae*. Our findings are similar to those of Pathmanathan *et al.*[18], who found increased sensitivity of PCR for detection of *S.* Typhimurium in direct stool samples from 1200 cfu PCR^{-1} to 120 cfu PCR^{-1} and 1.2 cfu PCR^{-1} after 4 and 6 h pre-enrichment of stool samples respectively. Further, the results showed that DNA templates from all the *Salmonella* isolates yielded 942, 305, 470 and 543 bp amplicons for *inv*A, *inv*H, *sop*B and *stn* genes respectively, while 254, 609 and 185 bp products (Figure 1) for *sop*E, *sef*C and *pef*A respectively, were produced by strains which harboured these genes. No amplicons of corresponding sizes were detected in case of non-*Salmonella* strains, indicating the specificity of the assay. Therefore, this assay demonstrates 100% specificity since all the desired amplicons were produced in *Salmonella* isolates, while no amplicon was produced from any of the non-*Salmonella* strains tested.

All the field isolates of corresponding serovars from the same source were found to have the same virulence gene profile in the m-PCR assay. The m-PCR assay developed in the present study was able to concurrently amplify more than one common as well as uncommon virulence locus in a single reaction. Since *inv*H, *stn* and *sop*B genes were found in all the isolates, they can be used as alternative target genes for direct detection of *Salmonella* from biological sources. This assay provides a rapid and reliable detection method for detection of *Salmonella* isolates with pathogenic potential and to compare their virulence gene profiles.

Both conventional PCR and m-PCR could detect presence of *Salmonella* Typhimurium in the spiked samples (faeces and bacterial growth). The inoculated samples enriched in tetrathionate broth and sub-cultured on BGA plates also yielded positive results in three of the five spiked samples containing 1 cfu ml^{-1} after 5 and 10 h of incubation, whereas PCR results were positive for all concentrations of *Salmonella* Typhimurium (in cfu ml^{-1} or cfu PCR^{-1}). Khan *et al.*[30] found *Salmonella*-positive results in food-spiked samples containing 1 cfu ml^{-1} after 10 h of incubation, implying that the m-PCR assay developed is more sensitive for detection of the target organism than the conventional culture method.

Conflict of interest: Authors declare that there is no conflict of interest with anyone in respect of the present study.

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