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Full Length Research Paper

Detection of genus *Salmonella* and serogroups A, B, C1, D and (*Vi*) capsular producing strains using multiplex polymerase chain reaction (PCR) method from stool

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In many countries, *Salmonella* is the leading cause of food-borne outbreaks and infections. A multiplex PCR (mPCR) for the detection of genus *Salmonella* and serogroups A, B, C1, D and capsular (*Vi*) producing strains from swabs of stool samples was developed. In the mPCR, primers for invasion (*invA*), O (*prt*, *tyv*, *rfbj*, *wzxC1*) and *Vi* antigen genes (*Vi*) and internal amplification control primers were used. The results showed that all tested *Salmonella* serotypes were accurately identified by the assay, without nonspecific amplification except *Salm*. derby and *Salm*. saint Paul. Representative serogroups were used to artificially inoculate stool samples. The different serogroups were detected by mPCR after overnight pre-enrichment of stool swab in buffered peptone water with a detection limit of *Salmonella* cell suspension of 4 cfu/ g stool. The developed mPCR assay provides specific detection of genus *Salmonella* and serogroups A, B, C1, D and *Vi* positive strains directly from stool swabs. The developed method for *Salmonella* serogroup identification is rapid, easy and less subjective methods. This could be of great use by any facility that lacks the expensive typing sera and expertise needed for conventional serotyping.

Key words: *Salmonella*, multiplex PCR, stool, molecular serotyping, somatic antigen.

INTRODUCTION

Salmonella enterica is one of the major bacterial agents that cause foodborne infections in humans all over the world (Herikstad et al., 2002). *Salmonella* detection by conventional bacteriological methods is laborious and time consuming (Swaminathan and Feng, 1994). The Kauffmanne White serotyping scheme for designation of *Salmonella* serotypes is used by most laboratories for the characterization of *Salmonella* isolates. The *Salmonella* serotype is determined on the basis of somatic (O) antigen which determines the group and flagellar (H) antigen which determines the serotype (Popoff 2001).

Several molecular methods based on the amplification of DNA, such as multiplex PCR, have been developed for the detection of *Salmonella* serotypes (Luk et al., 1993; Lim et al., 2003; Alvarez et al., 2004; Kim et al., 2006; Herrera et al., 2007; Levy et al., 2008; Lim and Thong 2009). Rahn et al. (1992) reported that the *invA* primer, targeting the invasion gene, was able to discriminate between *Salmonella* and non-*Salmonella* species. O antigen is a specific polysaccharide; the genes for O-antigen are grouped together on the chromosome in a gene cluster called *rfb* (Bastin and Reeves, 1995). The *rfb* genes encode the nucleotide sugar biosynthesis pathways and the transferases necessary for the assembly of polysaccharide O antigen (Herrera et al., 2007). The basis of the variation in O antigen structure is represented by the different types of sugar present, the arrangement of sugars, the addition of branch sugars, and modifying side groups; such variation is used to design serogroup specific probes (Luk et al., 1993). The production of *Vi* antigen is controlled by two chromosomal loci, *viaA* and *viaB*. The *vi*aB consists of the structural genes specific for *Vi*

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antigen expression (Hashimoto et al., 1993).

The main objective of this work was to describe a multiplex PCR that enables identification of genus *Salmonella* and identify serogroup A, B, C1, D and *Vi* strains and applying this method on the detection of *Salmonella* from stool.

MATERIALS AND METHODS

Bacterial strains

The *Salmonella* serotypes used in this study are listed in Table 1. These strains were obtained from the American Tissue Culture Collection (ATCC) and the Egyptian Central Health Laboratories (ECHL). Six enteric non *Salmonella* strains; *Klebsiella pneumoniae, Escherichia coli O157, Proteus mirabilis, Shigella flexneri, Enterobacter cloacae* and *Citrobacter freundii*, obtained from ECHL, were used to evaluate the specificity of the multiplex PCR reaction. The cultures were grown in LB broth and LB agar (Difco, Becton Dickinson, Sparks, MD, USA) at 37°C. Bacterial viable counts were obtained by plating 100 µl of each dilution on tryptic soy agar (Difco, Becton Dickinson, Sparks, MD, USA) plates and incubating overnight at 37°C.

DNA extraction

Crude DNA was prepared from 1 ml of bacterial suspension in phosphate buffer saline (PBS) adjusted to OD550 0.125. The bacterial suspension was centrifuged at 18,000 x g for 3 min. The pellet was dissolved in 500 µl double distilled water (DDW), boiled at 100°C for 15 min and cooled on ice for 5 min. The crude lysates were centrifuged at 18,000 x g for 10 min and 5 µl supernatant was used as template in the PCR reactions. Purified DNA was prepared using Generation column capture kit (Qiagen, Hilden, Germany).

Primers and internal amplification control

A primer pair targeting the invasion gene was used to detect genus *Salmonella* (Rahn et al., 1992). Primers for O-serogrouping multiplex PCR, specific for *Salmonella* serogroups A, B, C1 and D and positive strains were used (Hirose et al., 2002; Lim et al., 2003; Herrera et al., 2007; Levy et al., 2008; Lim and Thong, 2009). Internal control primers (P1-P2) were incorporated to avoid false negative results (Table 2). All primers used in the study were synthesized by Midland Certified Reagent Company, Inc, Midland, Texas, USA.

Multiplex PCR amplification and analysis of PCR products

Multiplex PCR was performed in a final volume of 25 µl in a Techne Touchgene Gradient thermal cycler (Duxford, Cambridge, United Kingdom). The optimized PCR mixture consisted of 0.1 µM F-*invA* and R-*invA* , 0.4 µM *F-prt*, *R-prt*, *F-rfbJ*, *R-rfbJ*, *F-tyv* and *R-tyv* , 1 µM *F-Vi*, *R-Vi*, *F-wzxC1* and *R-wzxC1*, 0.3 µM P1 and P2, 0.25 mM dNTPS, 3 mM MgCl2, 2.5 µg bovine serum albumin (BSA) and 2 units Taq DNA polymerase (Fermentas, EU) in 1x PCR buffer. The optimized cycling parameters of the mPCR consisted of denaturation at 95°C for 3 min, followed by 25 cycles 95°C for 30 s, 59°C for 30 s, 72°C for 60 s and a final extension at 72°C for 8 min. PCR product fragments were analyzed in 2% (w/v) agarose gel by electrophoresis using 1x TAE buffer and visualized by staining with ethidium bromide. Fragment size was determined by comparison with Gene-Ruler 100 bp DNA ladder (Fermentas, EU). ethidium bromide. Fragment size was determined by comparison with Gene-Ruler 100 bp DNA ladder (Fermentas, EU).

Sensitivity of the multiplex PCR

The sensitivity of the mPCR was evaluated using 10-fold dilutions in PBS starting from 1.5 x 10^3-1.5 x 10^7 cfu/ ml from *Salm. paratyphi*, *Salm. typhimurium*, *Salm. infantis*, *Salm. enteritidis* and *Salm. typhi* representing serogroups A, B, C1, D, respectively. Crude DNA was extracted from each dilution, and used as template in mPCR. The purified DNA template was prepared from representative strains using Generation column capture kit, as stated above. The purified DNA was quantified in the extracts using a Jenway 6800 Double-Beam spectrophotometer (Bibby Scientific Ltd, Staffordshire, UK).

Determination of specificity of the multiplex PCR

The specificity of the mPCR was checked with DNA prepared from the 25 *Salmonella* strains shown in Table 1 and the six non *Salmonella* enteric strains.

Sequence analysis

Six PCR products obtained with the primers representing each tested serogroup, *Vi* positive, and *invA* genes were sequenced to validate their identities. PCR products were purified using QIAquick PCR purification Kit (Qiagen, Hilden, Germany) and sequenced using ABI PRISM 310 sequencer (Clinilab, Cairo, Egypt).

Detection of *Salmonella* from spiked stool samples

Eleven stool specimens from a four year old healthy child (1 g each) were suspended in 9 ml PBS, and challenged with 10-fold dilutions from each representative strain to have a final bacterial count ranging from 3.5 x 10^{-2} - 3.5 x 10^8 cfu / ml. DNA was extracted from artificially spiked stool samples by two methods. In the first method, DNA was extracted directly from 1 ml stool sample using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). In the second method, swabs soaked with the spiked faecal samples were used to inoculate 10 ml buffered peptone water (BPW) and incubated overnight at 37°C. The overnight swab cultures were centrifuged at 10000 x g for 8 min at 4°C. The formed pellets were washed with one ml PBS, resuspended in 200 µl DDW and used for DNA extraction using QIAamp DNA Stool Mini Kit.

RESULTS

Multiplex PCR

Multiplex PCR was optimized by using different primers concentrations, Mg^{2+} concentration (1.5 mM - 3.5 mM) and addition of 2.5 µg BSA. Gradient annealing temperatures from 50 to 61°C were tested before selecting 59°C. Agarose gel of the PCR products is shown in Figure 1.

Sequence analysis

DNA sequence analysis (DNA blast search) of the PCR product of each of the six tested primer pairs showed high identity levels ranging from 97to 99% to the Gen-Bank sequence database, confirming the specificity of the primers.

Table 1. The band pattern of the optimized mPCR challenged with the tested *Salmonella* serotypes

Salmonella serotype	O group	Source	Number of strain	*invA*	*prt*	*tyv*	*rfbj*	*wzxC1*	*Vi*	*P1*
Paratyphi A	A	ATCC 13076	1	+	+	-	-	-	-	+
Paratyphi A	A	ECHL	1	+	+	-	-	-	-	+
Agona	B	ECHL	3	+	-	-	+	-	-	+
Typhimurium	B	ATCC 14028	1	+	-	-	+	-	-	+
Typhimurium	B	ECHL	2	+	-	-	+	-	-	+
Haifa	B	ECHL	1	+	-	-	+	-	-	+
Derby	B	ECHL	2	+	-	-	-	-	-	+
Saint Paul	B	ECHL	2	+	-	-	-	-	-	-
Infantis	C1	ATCC 15697	1	+	-	-	-	+	-	+
Infantis	C1	ECHL	1	+	-	-	-	+	-	+
Virchow	C1	ECHL	1	+	-	-	-	+	-	+
Bardo	C2	ECHL	1	+	-	-	-	-	-	+
Kentucky	C2	ECHL	2	+	-	-	-	-	-	+
Hadar	C2	ECHL	1	+	-	-	-	-	-	+
Newport	C2	ECHL	1	+	-	-	-	-	-	+
Enteritidis	D	ATCC 9150	1	+	+	+	-	-	-	+
Enteritidis	D	ECHL	1	+	+	+	-	-	-	+
Typhi	D	ECHL	2	+	+	+	-	-	+	+

Table 2. Multiplex PCR primers sequence, the target serogroup detected and the expected band size.

Primer	Target group	Primer sequence 5´ to 3´	Amplicon size (bp)	Source
F-prt	A, D	CTTGCTATGGAAGACATAACGAACC	256	M29682
R-prt		CGTCTCCATCAAAAGCTCCATAGA		
F-tyv	D	GAGGAAGGGAAATGAAGCTTTT	614	M29682
R-tyv		TAGCAAACTGTCTCCCACCATAC		
F-rfbJ	B	CCAGCACCAGTTCCAACTTGATAC	662	X56793
R-rfbJ		GGCTTCCGGCTTTATTGGTAAGCA		
F-wzxC1	C1	CAGTAGTCCGTAAAATACAGGGTGG	483	M84642
R-wzxC1		GGGGCTATAAATACTGTGTTAAATTCC		
F-vi	*Vi*	GTTATTCAGCATAAGGAG	439	D14156
R-vi		CTTCCATACCACTTTCCG		
F-invA	*InvA*	GTGAAATTATCGCCACGTTCGGGCAA	284	U43273
R-invA		TCATCGCACCGTCAAAGGAACC		
P1	*oric*	TTATTAGGATCGCGCCAGGC	163	Malorny *et al*, 2003
P2		AAAGAATAACCGTTGTTCAC		

Sensitivity of the multiplex PCR

The minimum limit of bacterial count detected in pure cultures, as well as the minimum amount of DNA detected by the mPCR for the six representative primers are shown in (Table 3).

Specificity of the multiplex PCR

The band pattern of the optimized mPCR, challenged with 25 *Salmonella* serotypes is reported in Table 1. All tested *Salmonella* serotypes showed the 284 bp band of *invA* gene which was undetected with the other tested six non *Salmonella* strains (Figure 1). Each serogroup was identified by its specific band pattern: serogroup A- 256 bp band, serogroup B - 662 bp band, serogroup C1- 483 bp band, serogroup D – 256 bp and 615 bp bands. *Salm*. typhi from serogroup D, which carries a *Vi* capsular antigen, showed 3 bands with corresponding sizes of 256 bp, 439 bp and 615 bp. A percentage of 100 % of the tested *Salmonella* strains (n=25), as well as serogroup A (n=2), C1 (n=3), D (n=4) and *Vi* Ag (n=2) and 64% of serogroup B (n=11) was correctly identified by the optimized mPCR. The specificity of the primers was tested

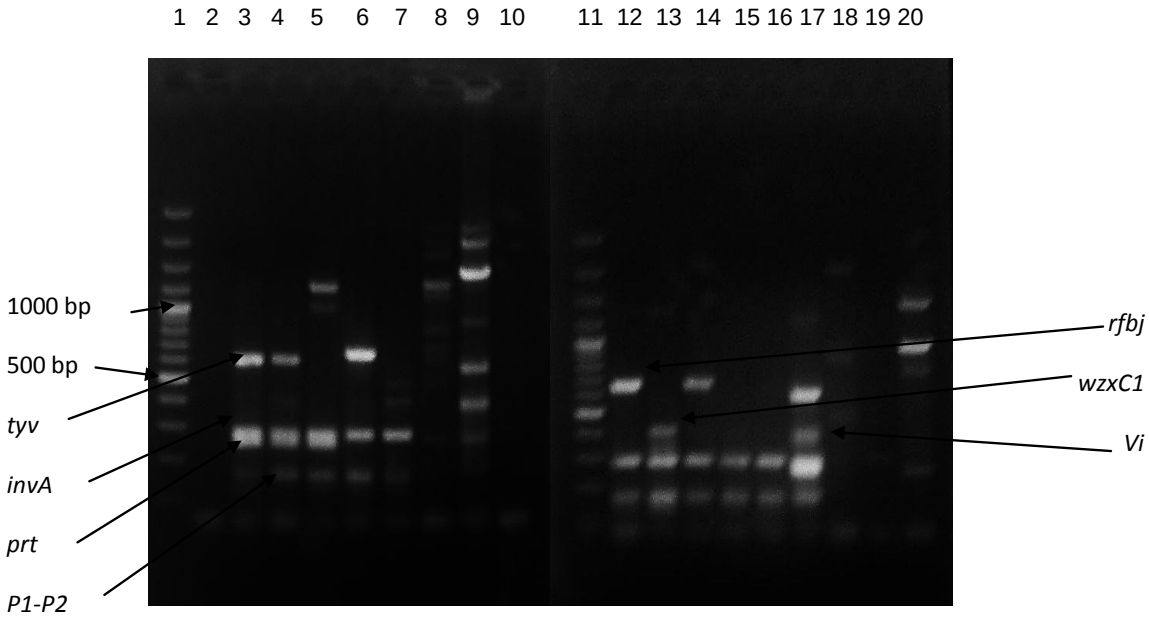

Figure. 1: Agrose gel electrophoresis of PCR products. Lane 1 and 11 show the profile of 100 bp marker. Lane 2 is a negative control reaction. The PCR was carried out with DNA obtained from *Salm. enteritidis* (lane 3, 4), *Salm. paratyphi A* (lane 5), *Salm. typhimurium* (lane 6), *Salm. infantis* (lane 7), *Enterobacter cloacae* (lane 8), *Citrobacter freundii* (lane 9), *Proteus mirabilis* (lane 10), *Salm. agona* (lane 12), *Salm. virchow* (lane 13), *Salm. haifa* (lane 14), *Salm. hadar* (lane 15), *Salm. bardo* (lane 16), *Salm. typhi* (lane 17), *Escherichia coli O157* (lane 18), *Shigella flexneri* (lane 19) and *Klebsiella pneumoniae* (lane 20).

Table 3. Minimum limit of bacterial count detected in pure cultures and in stool and the minimum amount of DNA detected by mPCR

Gene	Bacterial count limit in mPCR		DNA limit in PCR reaction
	Pure culture	Stool swabs after pre-enrichment in BPW	
invA	$4.75x10^4$cfu	4 cfu/ g stool	≤ 0.1 ng
Prt	$9.2x10^5$cfu	35 cfu/ g stool	1.3ng
Tyv	$9.2x10^4$cfu	4 cfu/g stool	0.312 ng
ViaB	$9.2x10^5$cfu	35 cfu/g stool	1.3 ng
rfbJ	$1.6x10^5$ cfu	4 cfu/g stool	0.225 ng
wzxC1	$4.75x10^6$cfu	$3.5x10^3$cfu /g stool	7.9 ng

with non-targeted serogroup C2 and non *Salmonella enterica* microorganisms; these primers showed no cross-reactivity. The PCR amplified an amplicon of 163 bp by P1-P2 primer and this was present in all tested *Salmonella* serotypes.

Sensitivity of mPCR with artificially spiked stool samples

Genus *Salmonella* was detected by the mPCR either directly from stool with a minimum bacterial count detection limit of 3.5×10^4 cfu/g stool or from stool swabs after overnight pre-enrichment in BPW with a minimum bacterial count detection limit of 4 cfu/g stool. The bacterial count detection limit of the mPCR for the different tested genes from pre-enriched stool swabs are recorded in (Table 3).

DISCUSSION

Herrera et al. (2007) had developed a multiplex PCR identifying serogroups B, C1, C2, D and E. In the O-grouping multiplex PCR developed by Levy et al. (2008), only serogroups A, B, D and *Vi*-positive strains were identified. Lim and Thong (2009) combined both these systems to cover a wider range of major serogroups A, B, C1, D and E as well as *Vi*-positive strains. In this study, a multiplex PCR assay was developed for the rapid detection of genus *Salmonella* (*invA*) and the most prevelant serogroups in Egypt, as isolated by ECHL, namely A, B,

C1, D and *Vi* producing strains from stool. The assay targeted the amplification of six genes *invA*, *prt*, *tyv*, *rfbJ*, *wzxC1* and *Vi* to represent the different tested serotypes. As expected, the sequences homologous to *invA* gene have been detected in all tested *Salmonella* serotypes with no cross reactivity with other enteric non *Salmonella* strains. This data correlates with that obtained by Chiu and Ou 1996. Although, the *prt* and *tyv* genes are present in serogroups A and D, the *tyv* gene of serogroup A has one nucleotide base deletion difference from *tyv* of serogroup D which creates an early codon stop in serogroup A, with the result that only paratose is incorporated in its O-antigen (Herrera et al., 2007). Based on this fact, a *tyv* band was observed with serogroup A with all tested annealing temperatures (gradient 50 -58°C) and was absent at higher annealing temperatures 59°C. This data was in contrast to the annealing temperatures, previously reported by Herrera et al. (2007) (58°C), Levy et al. (2008) (55°C) and Lim and Thong (2009) (50°C). In order to increase the specificity of the reaction, an annealing temperature of 59°C was selected. A specificity of 100% was detected by *wzxC1* and *viab* primers, as bands 622 bp and 439 bp, respectively were observed from all tested C1 and *Vi* positive strains, as reported in similar studies by Herrera et al. (2007), Levy et al. (2008) and Lim and Thong (2009). The current mPCR identified only 64% of tested serogroup B *Salmonella*, agona (n=3), typhimurium (n=3), haifa (n=1) and failed to identify *Salm*. derby (n=2) and *Salm*. saint Paul (n=2) despite being correctly identified as genus *Salmonella*. These two serotypes were not previously tested by Levy et al. (2008) and Lim and Thong (2009). The mPCR showed no cross reactivity with other tested serotypes of *Salmonella* or non *Salmonella* tested strains. The use of an internal amplification control (IAC) in diagnostic PCR is becoming mandatory (Hoorfar et al., 2003). In this study, the P1-P2 primer pair targeting the oriC gene was included as an internal control in all multiplex reactions. In this study, the optimum mPCR was prepared with $MgCl_2$ and BSA concentrations of 3 mM and 0.1 µg/ µl, respectively and an annealing temperature of 59°C; in contrast to that previously reported in similar studies by Herrera et al. (2007), Levy et al. (2008) and Lim and Thong (2009).

The most common problem in using a PCR assay for direct detection of an organism in faeces is that it contains substances such as bilirubin and bile salts that are inhibitory to PCR (Stone et al., 1994; Chiu and Ou, 1996). This can be eliminated by either DNA extraction or by enrichment of the faecal samples in a suitable broth prior to PCR (Dutta et al., 2001). In this study, the minimum bacterial count detected for genus *Salmonella* by the optimized mPCR directly from stool is 3.5×10^4 cfu / g stool, other tested genes were detected at higher levels (data not shown). This is comparable to the 7×10^4 cfu / ml stool detection limit obtained in a similar study by Trafny et al. (2006). However, the minimum bacterial count detected by the mPCR after an overnight pre-enrichment of a stool swab in BPW is 4 cfu / g stool, with an improve in the bacterial count detection limit by more than 1000 folds.

Traditional serotyping is a time-consuming process; the incorporation of these molecular tools for the serotyping of *Salmonella* has enabled the combining of both molecular and traditional methods, and therefore has increased the capacity of serotyping (Herrera et al., 2007). The use of this developed mPCR technique is straight forward, serogrouping could be carried out using a minimal number of reactions, and products can be detected easily by gel electrophoresis. It is reliable and reproducible as the interpretation of the results is not subjective. The technique is also fast promoting the detection of *Salmonella* and the serogroups A, B, C1, D and *Vi* positive strains in less than 24 h in contrast to the traditional methods followed by serotyping. To cover a wider range of tested serogroups, primers targeting serogroups C2 and E could be incorporated for developing further mPCR.

In conclusion, the results obtained in this study indicate that the multiplex PCR test is a sensitive, reliable, specific, and highly effective diagnostic tool for the simultaneous identification of *Salmonella* and its serogroups A, B, C1, D and *Vi* positive strains directly from stool. This system could greatly reduce our reliance upon the costly and tedious conventional serotyping.

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