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Multiplex TaqMan Real-Time PCR (qPCR) Assay Targeting *prot6E* and *invA* Genes for Fast and Accurate Detection of *Salmonella* Enteritidis

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1. Introduction

Salmonella is an important foodborne pathogen causing significant public health concern, both domestically and internationally (Tirado and Schmidt, 2001; Scallan et al., 2011). According to the latest CDC report *Salmonella* infections affect millions of people every year accounting for 11%, 35% and 28%, of illnesses, hospitalizations and deaths, respectively of the total U.S. foodborne diseases caused by all known foodborne pathogens (Scallan et al., 2011). Among those non-typhoid salmonellosis, *S. Enteritidis* (SE) has emerged as a major egg-associated pathogen. SE transmission to humans has been linked mainly to consumption of contaminated foods containing undercooked eggs (Rabsch et al., 2000). Fresh shell-eggs can be contaminated easily with SE through cracks in the shell by contact with chicken feces or by transovarian infection (Snoeyenbos et al., 1969). Consequently, the increase of consumption of shell eggs and egg products per capita in the United States to approximately 249 eggs per year (American Egg Board, 2008) may have contributed, in part, to increases in foodborne outbreaks (Altekruse et al., 1997), including a large multistate SE outbreak of SE outbreak associated with eggs in the US in 2010.

Traditional culture methods for SE detection from shell eggs and liquid whole eggs consist of a series of steps including non-selective pre-enrichment, selective enrichment, and selective/differential plating, and finally biochemical and serological confirmation. The traditional microbiological method for SE isolation from liquid eggs is described in detail in Chapter MLG 4.05 "Isolation and Identification of *Salmonella* from Meat, Poultry, Pasteurized Egg and Catfish Products" by the United States Department of Agriculture (USDA) (http://www.fsis.usda.gov/PDF/MLG_4_05.pdf). This method is labor intensive and takes about one weeks to complete the analysis. Consequently, a need exists for the development and validation of faster screening and detection methods for this pathogen in eggs.

The use of PCR or real time PCR (qPCR) for specific pathogen detection in foods has increased in recent years. They are fast and reliable tools for the testing of contaminated foods and had helped in preventing outbreaks. In recent years, numerous methods based on *Salmonella* DNA detection (e.g. *invA* gene) either by conventional or real-time PCR have been developed

(Krascsenicsova et al., 2008; Malorny B et al., 2003; Wolffs et al., 2006). qPCR is faster, is more sensitive than conventional PCR, and provides real-time data avoiding the use of gels (Valasek and Repa, 2005). In particular, the *invA* gene represents a good candidate for *Salmonella* detection as it is present in all pathogenic serovars described to date (Rahn et al., 1992; Boyd EF et al., 1997). The product of this gene is essential for the organism’s ability to invade mammalian cells and subsequently cause disease (Galan and Curtiss, III, 1991; Galan JE et al., 1992). In the case of SE in specific, several PCR and isothermal methodologies has also been developed targeting different genes (Seo et al., 2004; Malorny et al., 2007a; O'Regan et al., 2008; Hadjinicolaou et al., 2009). Although isothermal amplification techniques has some advantages over qPCR, such as increased detection limit and lower cost; still has the disadvantage that a single target can be used at a time and lacks internal control for monitoring possible inhibitors of the reaction that might exist in the food matrix analyzed.

In the present study we developed a fast and accurate qPCR assay for the specific detection of SE in eggs. This qPCR contained primers and probes to detect three different targets: the *invA* gene (*Salmonella* genus specific), the *prot6E* gene (SE specific), and the internal amplification control (IAC). A foreign internal amplification control (IAC) was incorporated into the assay with the aim of detecting potential inhibitors present in the matrix analyzed (eggs). *Salmonella* spp. detection in foods is usually achieved after food samples pre-enrichment approaches using overnight incubation (Feder et al., 2001). Consequently, this method described herein is intended as an initial screening of 24 h pre-enrichments for the presence of *Salmonella* in eggs. In turn, this method will dramatically decrease the time and effort required during standard microbiological testing, since only positive pre-enrichment samples will be processed further.

2. Materials and methods

2.1 Bacterial strains and media

Eleven *Salmonella enterica* serovar Enteritidis (SE) strains (CDC 2010K_1543, 13-2, SE12, 18579, 18580, 22689, SE10, SE26, 17905, SE22, and CDC_2010K_1441) (Table 1), were employed in this study for artificial contamination of eggs. Strain CHS44 was employed for determining the detection limit of the real-time PCR (qPCR) assay. These strains are from

Strain	Phage type	Location	Source
SE12	14b	ME	Egg follicle
18579	4	Mexico	poultry
18580	4	Mexico	poultry
22689	8	MD	Chicken breast
SE10	8	ME	Chicken ovary
SE26	13	TX	Chicken viscera
13-2	13	N/A	Chicken
17905	13a	N/A	Chicken
SE22	13a	ME	Poultry environment
CDC_2010K_1441	N/A	N/A	Egg outbreak USA 2010
CDC_2010K_1543	N/A	N/A	Egg outbreak USA 2010

Table 1. Characteristics of *S. Enteritidis* strains used in this study for artificial contamination of eggs.

the FDA, Center for Food Safety and Applied Nutrition (CFSAN), Division of Microbiology’s culture collection. Strains were grown overnight in Luria-Bertani (LB) medium at 35°C with shaking (250 rpm). The inclusivity and exclusivity of the qPCR assay for SE was demonstrated with 186 SE (Table 2) and 97 non-SE strains belonging to the FDA’s collection (Table 3). Further specificity was demonstrated with 32 non-*Salmonella* species (48 strains) from very closely related genera (Table 4).

S. Enteritidis strains	qPCR multiplex		
	<i>invA</i> qPCR result	<i>prot6E</i> qPCR result	IAC qPCR result
54-5431	+	+	+
76-574	+	+	+
19755	+	+	+
22568	+	+	+
53-407	+	+	+
50-5306A	+	+	+
81-2625	+	+	+
78-1757	+	+	+
77-0424	+	+	+
36951	+	+	+
75-2325	+	+	+
60-2506	+	+	+
74-991	+	+	+
77-3493	+	+	+
76-2651	+	+	+
62-1976	+	+	+
77-1427	+	+	+
77-2659	+	+	+
75-199	+	+	+
76-2969	+	+	+
17912	+	+	+
75-1450	+	+	+
78-2938	+	+	+
31952	+	+	+
36388	+	+	+
76-1594	+	+	+
50-3079	+	+	+
50-5646	+	+	+
75-970	+	+	+
576709	+	+	+
639016-6	+	+	+

S. Enteritidis strains	qPCR multiplex		
	<i>invA</i> qPCR result	<i>prot6E</i> qPCR result	IAC qPCR result
607307-2	+	+	+
635290-58	+	+	+
640631	+	+	+
60738-9	+	+	+
629163	+	+	+
60-7307-6	+	+	+
622731-39	+	+	+
607308-16	+	+	+
607308-19	+	+	+
8a	+	+	+
98	+	+	+
415	+	+	+
13-1	+	+	+
23b	+	+	+
sz26	+	+	+
416 (pt4)	+	+	+
435 (pt4)	+	+	+
sz6	+	+	+
sz9	+	+	+
418	+	+	+
23a	+	+	+
pt23	+	+	+
421	+	+	+
sz10 (pt8)	+	+	+
436	+	+	+
414 (pt4)	+	+	+
419	+	+	+
CHS14	+	+	+
sz15 (pt8)	+	+	+
420	+	+	+
sz12	+	+	+
sz22	+	+	+
434	+	+	+
CHS44	+	+	+
426	+	+	+
13-3	+	+	+
60481	+	+	+

S. Enteritidis strains	qPCR multiplex		
	<i>invA</i> qPCR result	<i>prot6E</i> qPCR result	IAC qPCR result
chs15	+	+	+
chs39	+	+	+
60494	+	+	+
60562	+	+	+
13-2	+	+	+
30663	+	+	+
22689 (pt8)	+	+	+
18570	+	+	+
23711	+	+	+
22705	+	+	+
22599	+	+	+
22706	+	+	+
23698	+	+	+
23703	+	+	+
22581	+	+	+
22600	+	+	+
22606 (pt8)	+	+	+
22574	+	+	+
33944	+	+	+
22690 (pt8)	+	+	+
22619	+	+	+
22601	+	+	+
18580 (pt4)	+	+	+
18572 (pt4)	+	+	+
18671 (pt4)	+	+	+
22532	+	+	+
18577	+	+	+
18512	+	+	+
18575	+	+	+
17924	+	+	+
18511	+	+	+
18578	+	+	+
18579	+	+	+
17927	+	+	+
18510	+	+	+
17929	+	+	+
1793	+	+	+

S. Enteritidis strains	qPCR multiplex		
	<i>invA</i> qPCR result	<i>prot6E</i> qPCR result	IAC qPCR result
18568	+	+	+
18567 (pt4)	+	+	+
18518	+	+	+
13183	+	+	+
17912	+	+	+
18088	+	+	+
22621	+	+	+
17914	+	+	+
18514	+	+	+
17923 (pt8)	+	+	+
17921	+	+	+
17905	+	+	+
18509	+	+	+
17918	+	+	+
17919	+	+	+
17917	+	+	+
30661	+	+	+
18569	+	+	+
18081	+	+	+
22568	+	+	+
17930	+	+	+
22701	+	+	+
30658	+	+	+
18574	+	+	+
18516	+	+	+
18573	+	+	+
17931	+	+	+
22510 (pt8)	+	+	+
CDC_2010K_0895	+	+	+
CDC_2010K_0899	+	+	+
CDC_2010K_0956	+	+	+
CDC_2010K_0968	+	+	+
CDC_2010K_1010	+	+	+
CDC_2010K_1018	+	+	+
CDC_2010K_1441	+	+	+
CDC_2010K_1444	+	+	+
CDC_2010K_1445	+	+	+

S. Enteritidis strains	qPCR multiplex		
	<i>invA</i> qPCR result	<i>prot6E</i> qPCR result	IAC qPCR result
CDC_2010K_1455	+	+	+
CDC_2010K_1457	+	+	+
CDC_2010K_1543	+	+	+
CDC_2010K_1558	+	+	+
CDC_2010K_1559	+	+	+
CDC_2010K_1565	+	+	+
CDC_2010K_1566	+	+	+
CDC_2010K_1575	+	+	+
CDC_2010K_1580	+	+	+
CDC_2010K_1594	+	+	+
CDC_2010K_1725	+	+	+
CDC_2010K_1729	+	+	+
CDC_2010K_1745	+	+	+
CDC_2010K_1747	+	+	+
CDC_2010K_1791	+	+	+
CDC_2010K_1795	+	+	+
CDC_2010K_1808	+	+	+
CDC_2010K_1810	+	+	+
CDC_2010K_1811	+	+	+
CDC_2010K_1882	+	+	+
CDC_2010K_1884	+	+	+
SE12	+	+	+
SE26	+	+	+
SE22	+	+	+
CDC_07ST000857	+	+	+
CDC_08-0253	+	+	+
CDC_08-0254	+	+	+
02-0062	+	+	+
58-6482	+	-	+
59-365	+	-	+
54-2953	+	-	+
chs54	+	-	+
20036	+	-	+
20035	+	-	+
18845	+	-	+
32393	+	-	+
18685	+	-	+

S. Enteritidis strains	qPCR multiplex		
	invA qPCR result	prot6E qPCR result	IAC qPCR result
22558	+	-	+
20034	+	-	+
20037	+	-	+
23710	+	-	+
sz23	+	-	+
sz5	+	-	+
SE-10	+	-	+
Total (186)	186	170	186

Table 2. *Salmonella* strains used for testing SE inclusivity for the *prot6E/invA* multiplex TaqMan qPCR.

<i>Salmonella</i> subspecies and serovars	Strain Numbers	prot6E qPCR result	invA qPCR result	IAC qPCR result
<i>S. enterica</i> subsp. <i>enterica</i> (I)				
Typhimurium	14	-	+	+
I 4,[5],12:i:-	6	-	+	+
Typhimurium/DT104	4	-	+	+
Newport	1	-	+	+
Heidelberg	1	-	+	+
Typhi	1	-	+	+
4,5,12:b:-	1	-	+	+
Hadar	1	-	+	+
Brandenburg	1	-	+	+
Saphra	1	-	+	+
Rubislaw	1	-	+	+
Michigan	1	-	+	+
Urbana	1	-	+	+
Vietnam	1	-	+	+
Tornow	1	-	+	+
Gera	1	-	+	+
Fresno	1	-	+	+
Brisbane	1	-	+	+
Agona	1	-	+	+
Muenchen	1	-	+	+
Senftenberg	1	-	+	+
Muenster	1	-	+	+

<i>Salmonella</i> subspecies and serovars	Strain Numbers	<i>prot6E</i> qPCR result	<i>invA</i> qPCR result	IAC qPCR result
Montevideo	1	-	+	+
Johannesburg	1	-	+	+
Javiana	1	-	+	+
Inverness	1	-	+	+
Cubana	1	-	+	+
Cerro	1	-	+	+
Alachua	1	-	+	+
<i>S. enterica</i> subsp. <i>Salamae</i> (II)				
II 58:l,z13,z28:z6	1	-	+	+
II 47:d:z39	1	-	+	+
II 48:d:z6	1	-	+	+
II 50:b:z6	1	-	+	+
II 53:lz28:z39	1	-	+	+
II 39:lz28:enx	1	-	+	+
II 13,22:z29:enx	1	-	+	+
II 4,12:b:-	1	-	+	+
II 18:z4,z23:-	1	-	+	+
<i>S. enterica</i> subsp. <i>arizonae</i> (IIIa)				
IIIa 41:z4,z23:-	1	-	+	+
IIIa 40:z4,z23:-	1	-	+	+
IIIa 48:g,z51:-	1	-	+	+
IIIa 21:g,z51:-	1	-	+	+
IIIa 51:gz51:-	1	-	+	+
IIIa 62:g,z51:-	1	-	+	+
IIIa 48:z4,z23,z32:-	1	-	+	+
IIIa 48:z4,z23:-	1	-	+	+
<i>S. enterica</i> subsp. <i>diarizonae</i> (IIIb)				
IIIb 60:r:e,n,x,z15	1	-	+	+
IIIb 48:i:z	1	-	+	+
IIIb 61:k:1,5,(7)	1	-	+	+
IIIb 61:l,v:1,5,7	1	-	+	+
IIIb 48: z10: e,n,x,z15	1	-	+	+
IIIb 38:z10:z53	1	-	+	+
IIIb 60:r:z	1	-	+	+

<i>Salmonella</i> subspecies and serovars	Strain Numbers	<i>prot6E</i> qPCR result	<i>invA</i> qPCR result	IAC qPCR result
IIIb 50:i:z	1	-	+	+
<i>S. enterica</i> subsp. <i>houtenae</i> (IV)				
IV 50:g,z51:-	1	-	+	+
IV 48:g,z51:-	1	-	+	+
IV 44:z4,z23:-	1	-	+	+
IV 45:g,z51:-	1	-	+	+
IV 16:z4,z32:-	1	-	+	+
IV 11:z4,z23:-	1	-	+	+
IV 6,7:z36:-	1	-	+	+
IV 16:z4,z32:-	1	-	+	+
<i>S. enterica</i> subsp. <i>Indica</i> (VI)				
VI 6,14,25:z10:1,(2),7	1	-	+	+
VI 11:b:1,7	1	-	+	+
VI 6,7:z41:1,7	1	-	+	+
VI 11:a:1,5	1	-	+	+
VI 6,14,25:a:e,n,x	1	-	+	+
<i>S. enterica</i> subsp. <i>houtenae</i> (VII)				
IV 40:g,z51:-	1	-	+	+
IV 40:z4,z24:-	1	-	+	+
<i>S. bongori</i> (V)				
V 48:i:-	1	-	+	+
V 40:z35:-	1	-	+	+
V 44:z39:-	1	-	+	+
V 60:z41:-	1	-	+	+
V 66:z41:-	1	-	+	+
V 48:z35:-	1	-	+	+
Total	101			

The nomenclatural system used is based on recommendations from the WHO Collaborating Centre for reference and research on *Salmonella*, 9th edition 2007.

Table 3. *Salmonella* strains used for testing SE exclusivity for the *prot6E/invA* multiplex TaqMan qPCR assay.

Organism	No. of strains	<i>prot6E</i> qPCR result	<i>invA</i> qPCR result	IAC qPCR result
<i>Vibrio parahaemolyticus</i>	4	-	-	+
<i>V. vulnificus</i>	1	-	-	+
<i>Escherichia coli</i>	9 ^a	-	-	+
<i>Enterobacter cloacae</i>	1	-	-	+
<i>E. aerogenes</i> (ATCC 13048)	1	-	-	+
<i>Cronobacter sakazakii</i> (former <i>E. sakazakii</i>)	1	-	-	+
<i>Yersinia enterocolitica</i>	1	-	-	+
<i>Y. pseudotuberculosis</i>	1	-	-	+
<i>Hafnia alvei</i>	2	-	-	+
<i>Morganella morganii</i>	1	-	-	+
<i>Edwardsiella tarda</i>	1	-	-	+
<i>Klebsiella pneumoniae</i>	1	-	-	+
<i>Proteus vulgaris</i>	1	-	-	+
<i>Pseudomonas aeruginosa</i>	1	-	-	+
<i>Serratia marcesans</i>	1	-	-	+
<i>Aeromonas hydrophila</i>	1	-	-	+
<i>Citrobacter freundii</i>	1	-	-	+
<i>C. koseri</i> (ATCC 27028)	1	-	-	+
<i>Staphylococcus aureus</i>	1	-	-	+
<i>Streptococcus faecalis</i>	1	-	-	+
<i>Bacillus subtilis</i>	1	-	-	+
<i>B. cereus</i>	1	-	-	+
<i>Listeria monocytogenes</i>	1	-	-	+
<i>L. innocua</i>	1	-	-	+
<i>Shigella sonnei</i>	2	-	-	+
<i>S. flexneri</i>	2	-	-	+
<i>S. boydii</i>	2	-	-	+
<i>S. dysenteriae</i>	2	-	-	+
<i>Achromobacter spp.</i>	1	-	-	+
<i>Providencia stuartii</i> (ATCC 33672)	1	-	-	+
<i>Proteus mirabilis</i>	1	-	-	+
<i>P. hauseri</i> (deposited as <i>P.vulgaris</i>) (ATCC 13315)	1	-	-	+
Total	48			

^a Five *E. coli* classes (virotypes) that cause diarrheal diseases were included: strain 10009 (enterotoxigenic, ETEC); strains 10010, 10015, 10016, 10017 and 10012 (enteroinvasive, EIEC); strain 10023 (enterohemorrhagic, EHEC); strain 10035 (enteropathogenic, EPEC) and strain ATM395 (enteroaggregative, EAEC).

Table 4. Organisms employed to assess the specificity of the *prot6E/invA* multiplex *TaqMan* qPCR assay for *S. Enteritidis* detection.

2.2 Preparation of SE inocula

Cultures of individual SE strains were prepared by transferring a loopful for three consecutive 24-h intervals to 10 ml of tryptic soy broth (TSB, Difco, Becton Dickinson) at 35 °C. SE cells from an overnight broth culture were centrifuged at 3,000 × g for 15 min at 4 °C. The pellet was washed twice with sterile 0.1% peptone water and re-suspended in sterile 0.1% peptone water. Serial dilutions of the suspension were prepared in sterile 0.1% peptone water to obtain the desired cell populations. The cell number in the inoculum was determined by plating 100 µl dilutions (in sterile 0.1% peptone water) on TSA and incubating at 35 °C for 24 h.

2.3 Microbiological assay

All eggs were purchased from local grocery stores in College Park, MD. Analysis of liquid eggs was performed by following USDA procedure with some modifications. Shell eggs were broken by hands aseptically into sterile glass beakers. They were mixed well with a sterile stick by hands for about 2 minutes until it looked uniform. These liquid eggs were inoculated (day 1) at around 5 SE or at $> 10^4$ cells in 100 g. Each 100 g sample was placed into a 2-liter sterile glass beaker, mixed with 900 ml pre-enrichment broth. Five pre-enrichment broths were used for testing performance of pre-enrichments for SE recovery. They were TSB, TSB plus ferrous sulfate (TSB + Fe), universal pre-enrichment broth (UPB), nutrient broth (NB), and buffered peptone water (BPW). After 24 hr (day 2) pre-enrichment, 1 ml of each pre-enriched sample was transferred to 10 ml of selective enrichment media (Rappaport-Vassiliadis (RV) medium and Tetrathionate broth (TT) (Difco) and incubated for 24 h at 42 °C and 43 °C, respectively. On day 3, tube contents were vortexed for 10 sec, and 10 µl portions of the TT and RV media were streaked on bismuth sulfite (BS) agar, xylose lysine desoxycholate (XLD) agar, and Hektoen enteric (HE) agar and incubated at 35 °C for 24 h. On day 4, the plates were examined for the presence of typical *Salmonella* colonies. Typical colonies were confirmed with *Salmonella* agglutination test kit from BD.

2.4 Design of primers and standards for qPCR

All primers and probes (Table 5) employed in this study were purchased from IDT (Coralville, IA, USA). The targets for qPCR were *invA* gene and *prot6E* gene of SE. Primers and probes for *invA* assay were designed previously (Gonzalez-Escalona et al., 2009). Primers and probes for *prot6E* were designed using Beacon designer v.7 (PREMIER Biosoft, Palo Alto, CA). DNA from strain CHS44 was used to determine the *prot6E/invA* qPCR detection limit. DNA extraction was performed with the DNeasy kit as recommended by the manufacturer (QIAGEN). DNA concentration was determined using Qubit® 2.0 Fluorometer and Qubit™ dsDNA HS Assay Kit following manufacturer's instructions (Invitrogen). The numbers of copies of the qPCR standards were calculated by assuming average molecular masses of 680 Da for 1 nucleotide of double stranded DNA. The calculation was done with the following equation: copies per nanogram = $(NL \times 10^{-9}) / (n \times mw)$, where n is the length of the SE strain P125109 complete genome (4,685,848 bp), mw is the molecular weight per nucleotide, and NL is Avogadro constant (6.02×10^{23} molecules per mol).

Target	Name	Sequence (5'-3') ^a	Reference
qPCR primers			
invA	invA_176F	CAACGTTTCCTGCGGTACTGT	(Gonzalez-Escalona et al., 2009)
	invA_291R	CCCGAACGTGGCGATAATT	
prot6E	prot6E-NGE-f	GTAGGTAGCCAGTATAAATC	This study
	prot6E-NGE-r	TCGGTTTCATAATCATTCC	
IAC	IAC-f	CTAACCTTCGTGATGAGCAATCG	(Deer et al., 2010)
	IAC-r	GATCAGCTACGTGAGGTCCTAC	
Probes			
	invA_Tx_208	TX-CTCTTTCGTCTGGCATTATCGATCAGTACCA-BHQ2	(Gonzalez-Escalona et al., 2009)
	prot6E-NGE-FAM	FAM-CACCACAAT/ZEN/ATGCGAATGAACCGT -BHQ3	This study
	IAC-Cy5	Cy5-AGCTAGTCGATGCACTCCAGTCCTCCT-Iowa BlackRQ-Sp	(Deer et al., 2010)

Table 5. Primers and probes employed in this study to detect *prot6E/invA* by qPCR. TX – Texas Red.

2.5 qPCR and data analysis

The qPCR reactions were carried out using the Platinum® Quantitative PCR SuperMix-UDG kit according to the specifications of the manufacturer (Invitrogen). This kit is a ready to use cocktail consisting of a 2X Reaction Mix (Platinum® Taq polymerase, 40 mM Tris-HCl, 100 mM KCl, 6 mM MgCl₂, 0.4 mM of each dNTP, 0.8 of dUTP, uracil DNA glycosilase (UDG) and stabilizers). Reactions were scaled down to a final volume of 20 µl. Additional MgCl₂ was added to the master mix to a final concentration per tube reaction of 5 mM. Also additional Platinum® Taq polymerase was supplied in order to have 2.5 final units per reaction. Final concentrations of primers in the qPCR mix were 200 nM for *invA* and *prot6E*, and 100 nM for IAC, respectively. Probes were added to a final concentration of 150 nM. qPCR and data analysis was performed on a Mx3005P QPCR System (Agilent Technologies, Inc., Santa Clara, CA) real-time PCR machine. qPCR conditions were as follows: an initial cycle of 2 min at 50°C for UDG incubation, a second cycle of 2 min at 95°C to activate the hot-start Taq polymerase and 35 cycles of denaturation at 95°C for 15 secs, primer annealing and extension at 60°C for 30 secs (the acquisition of dyes Cy5, FAM and Texas Red were performed at the end of this cycle). Two microliters of DNA IAC (10 pg -3,0 * 10⁵ copies/2µl) was added to each qPCR reaction.

3. Results

3.1 Evaluation of the *prot6E/invA* multiplex qPCR TaqMan assay

The detection limit of the *prot6E/invA* qPCR was determined using 10-fold dilutions of DNA extracted from *S. enterica* Enteritidis strain CHS44. PCR primers specific for *prot6E* gene (*prot6E*-NGE-f and *prot6E*-NGE-r) and *invA* gene (*invA*_176F and *invA*_291R) were used (Table 5). Linear calibration curves with a correlation coefficient (R^2) of ≥ 0.99 and linear ranges of ≥ 5 orders of magnitude for both *prot6E* and *invA* were obtained (Fig. 1A and B). This corresponds to detection limits of about 40 genome copies for both *prot6E* and *invA* genes. The efficiency of the qPCR was ≥ 0.99 for both SE targets. The robustness of DNA IAC was observed for all dilutions tested (Fig. 1C). The inclusion of the DNA IAC (internal amplification control) did not affect amplification of either *Salmonella* gene target (Fig. 1C).

3.2 Specificity of the *prot6E/invA* qPCR TaqMan assay

The developed *prot6E/invA* qPCR assay showed 100% (186/186) and 91% (170/186) inclusivity for *invA* and *prot6E* target, respectively, after testing 186 SE strains (Table 2). The strains that rendered a negative result for presence of *prot6E* were: SE-10, 58-6482, 59-365, 54-2953, CHS54, 20036, 20035, 18845, 32393, 18685, 22558, 20034, 20037, 23710, sz23, and sz5. Furthermore, *prot6E/invA* qPCR showed 100% exclusivity, only SE was positive for *prot6E* target, while all *Salmonella* strains tested were positive for *invA* gene (Table 3). Specificity of the new multiplex *prot6E/invA* qPCR assay was examined by testing 48 non-*Salmonella* (Table 4), and was 100% specific for SE. These strains were chosen for specificity testing because many are close phylogenetic kin to the *Salmonellae* and, in several cases, are known to associate with the food supply. False negatives (inhibition of PCR reaction) were also ruled out through the use of a DNA internal amplification control (IAC).

3.3 Performance assessment of different pre-enrichment media for the recovery of SE from eggs using *prot6E/invA* qPCR and USDA microbial culture methods

The usefulness of the qPCR assay developed in this study for detecting SE in eggs was assessed by artificial contamination of eggs with SE. One hundred grams of pooled eggs were artificially contaminated with two different SE strains (CDC-2010K_1543 and 13-2) at high (10^6 CFU/100 g) and low (<10 CFU/100 g) levels (Table 6). We further tested the performance of 5 different pre-enrichment media for SE growth (BPW, TSB, TSB+Fe, NB, and UP). After 24 h, the pre-enrichments were used for detection of SE using both *prot6E/invA* qPCR and USDA *Salmonella* culture method (Chapter MLG 4.05 - "Isolation and Identification of *Salmonella* from Meat, Poultry, Pasteurized Egg and Catfish Products"; http://www.fsis.usda.gov/PDF/MLG_4_05.pdf). Un-inoculated egg samples were used as negative controls. One milliliter of pre-enrichment was boiled and used for qPCR amplification in triplicate. All artificially contaminated egg samples were positive for *Salmonella* using both *prot6E/invA* qPCR and the USDA methodologies (Table 6). We chose to show in the table only lower inoculation levels in order to highlight the sensitivity of this qPCR method. SE levels as low as 5 CFU/100 g were detected after 24 ± 2 h pre-enrichments. All pre-enrichment media showed fairly similar performances for SE recovery, save for NB which showed less growth after 24h, with SE levels 10-fold lower than other

media (Table 6). Absence of qPCR inhibitors was demonstrated by amplification of the IAC since IAC would not have been amplified had there been PCR inhibitors present in the samples analyzed (Table 6).

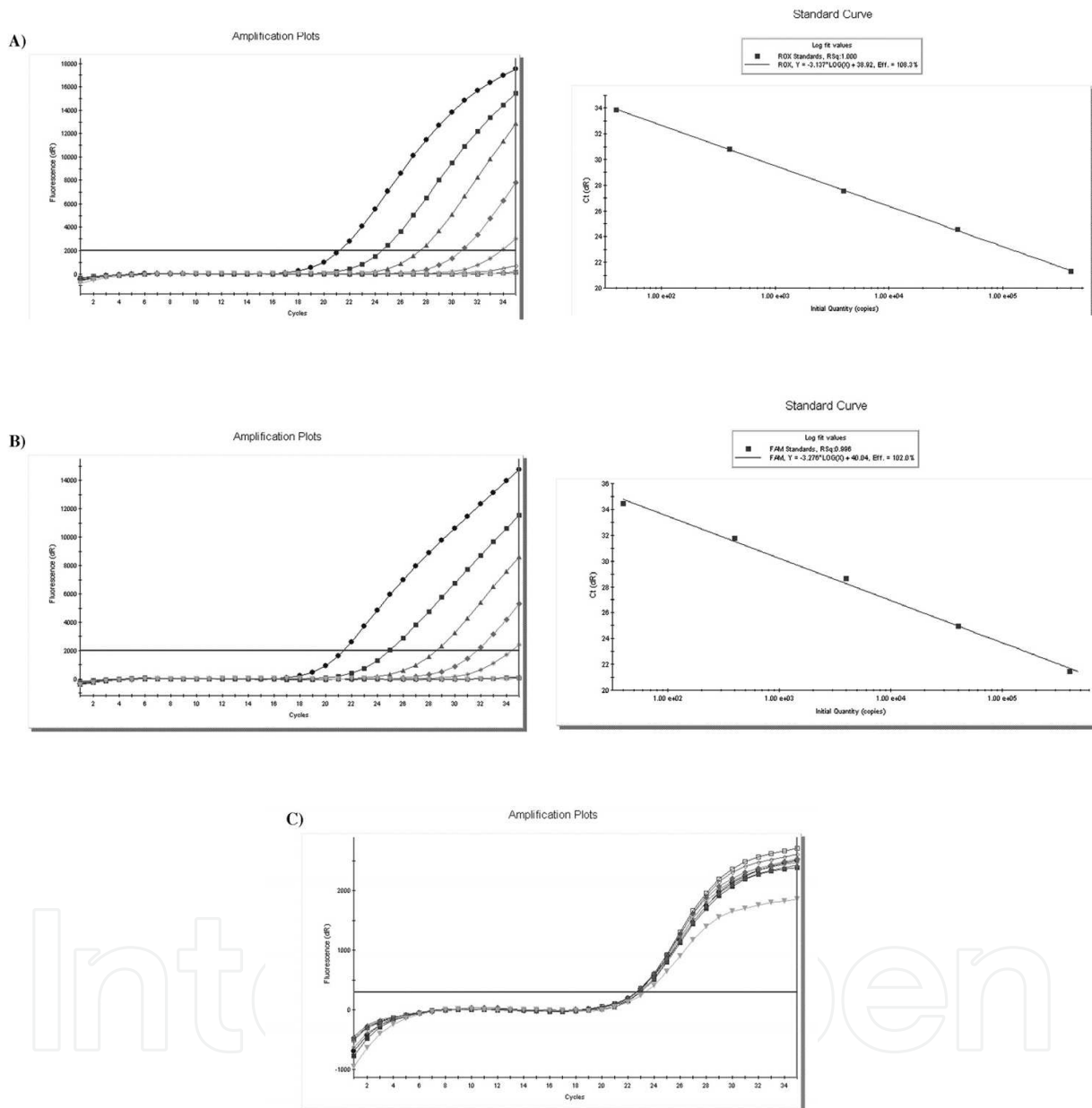


Fig. 1. Detection limit of the multiplex qPCR assay. Calibration curves were generated using 10-fold dilutions of CHS44 DNA (three replicates). A) Average *prot6E* amplification from dilutions 4.0×10^6 - 40 CHS44 genome copies/reaction tube (FAM channel). B) Average *invA* amplification from dilutions 4.0×10^6 - 40 CHS44 genome copies/reaction tube (ROX channel). C) Concurrent IAC amplification for each dilution (Cy5 channel). The C_q values were plotted against the nucleic acid target concentration (as copies per reaction for both DNA). The reaction efficiency (E) and R^2 values are also shown.

SE Strain	Pre-enrichment media	Minimum inoculation levels detected by USDA (CFU/100 g)	qPCR		
			<i>prot6E</i> (Cq)	<i>invA</i> (Cq)	IAC (Cq)
CDC-2010K_1543	BPW	5	+ (18.71 ± 0.79)	+ (19.98 ± 0.97)	+ (22.96 ± 0.41)
	NB		+ (22.69 ± 0.30)	+ (24.16 ± 0.38)	+ (22.31 ± 0.16)
	UP		+ (21.22 ± 0.55)	+ (20.12 ± 0.88)	+ (24.11 ± 1.30)
	TSB		+ (20.73 ± 0.90)	+ (22.22 ± 1.08)	+ (22.50 ± 0.42)
	TSB + Fe		+ (18.88 ± 0.30)	+ (20.32 ± 0.39)	+ (22.26 ± 0.66)
13-2	BPW	5	+ (17.70 ± 0.64)	+ (19.36 ± 0.72)	+ (22.69± 0.23)
	NB		+ (23.12 ± 1.13)	+ (24.39 ± 1.07)	+ (22.27± 0.32)
	UP		+ (19.63 ± 0.36)	+ (19.29 ± 0.79)	+ (23.83 ± 0.68)
	TSB		+ (20.24 ± 0.88)	+ (21.50 ± 0.62)	+ (22.42± 0.32)
	TSB + Fe		+ (19.43± 0.68)	+ (20.92 ± 0.82)	+ (22.20 ± 0.35)

Cq – Cycle quantification threshold, where the fluorescent is higher than the background.
+ = *Salmonella* positive by the method. In the case of IAC stands for positive signal for IAC.

Table 6. Pre-enrichment medium assessment for *Salmonella* Enteritidis (SE) recovery and detection using *prot6E/invA* multiplex qPCR and USDA culture method in pooled eggs artificially contaminated. Cq values are given in parentheses.

3.4 Application of the *prot6E/invA* qPCR assay for *Salmonella* Enteritidis detection in eggs artificially contaminated with different SE strains

After determining the effectiveness of the different pre-enrichment medias, we decided to employ TSB media as pre-enrichment for testing artificially contaminated eggs with 9 additional SE strains (Table 7). One of these strains lacked *prot6E* gene (SE-10). Eighteen pooled egg samples (100 g each) were artificially contaminated with high (~ 10⁶ CFU/100 g) and low (<10 CFU/100 g) levels of SE and were analyzed as mentioned previously. After 24h, all artificially contaminated egg pre-enrichments were used for detection of SE using both *prot6E/invA* qPCR and USDA *Salmonella* culture method. Un-inoculated egg samples resulted in negative results by both *prot6E/invA* qPCR and USDA *Salmonella* culture method. On the other hand, all artificially contaminated egg samples were positive for

Salmonella using both *prot6E/invA* qPCR and the USDA methodologies except for SE-10 strain which was negative for *prot6E* (Table 7). Contrary to what is shown in Table 6, we showed the results of both high and low inoculation levels Table 7. SE levels as low as 2 CFU/100 g were detected after 24 ± 2 h pre-enrichments. As mentioned previously for the pre-enrichment media, the absence of qPCR inhibitors was demonstrated by observing no inhibition of the amplification of the IAC in every sample (Table 7).

SE Strain	Inoculation levels detected by USDA (CFU/100 g)	qPCR (Cq)		
		<i>prot6E</i>	<i>invA</i>	IAC
SE12	-	-	-	+ (23.33 \pm 0.38)
	1.15 * 10 ⁴	+ (23.17 \pm 1.35)	+ (24.32 \pm 0.68)	+ (23.08 \pm 0.66)
	3	+ (23.32 \pm 0.72)	+ (24.34 \pm 0.96)	+ (23.72 \pm 1.10)
18579	-	-	-	+ (24.11 \pm 0.45)
	1.24 * 10 ⁴	+ (23.56 \pm 1.22)	+ (24.45 \pm 0.29)	+ (22.91 \pm 0.31)
	4	+ (24.47 \pm 0.76)	+ (24.72 \pm 0.81)	+ (23.57 \pm 1.17)
18580	-	-	-	+ (23.45 \pm 0.53)
	0.68 * 10 ⁴	+ (22.49 \pm 0.59)	+ (24.32 \pm 0.64)	+ (22.85 \pm 0.43)
	2	+ (22.51 \pm 0.72)	+ (23.97 \pm 0.60)	+ (22.70 \pm 0.14)
22689	-	-	-	+ (23.35 \pm 0.37)
	1.35 * 10 ⁴	+ (21.77 \pm 0.88)	+ (23.12 \pm 0.85)	+ (22.51 \pm 0.52)
	4	+ (23.83 \pm 0.91)	+ (24.83 \pm 0.81)	+ (24.42 \pm 1.04)
SE10	-	-	-	+ (23.30 \pm 0.36)
	1.32 * 10 ⁴	-	+ (23.20 \pm 0.43)	+ (23.01 \pm 0.45)
	4	-	+ (24.61 \pm 0.75)	+ (22.83 \pm 0.11)
SE26	-	-	-	+ (23.04 \pm 0.40)
	1.53 * 10 ⁴	+ (21.44 \pm 0.28)	+ (22.93 \pm 0.43)	+ (22.40 \pm 0.37)
	5	+ (23.70 \pm 0.91)	+ (24.76 \pm 0.69)	+ (23.23 \pm 0.48)
17905	-	-	-	+ (23.35 \pm 0.44)
	1.52 * 10 ⁴	+ (22.14 \pm 0.26)	+ (23.58 \pm 0.55)	+ (22.20 \pm 0.59)
	5	+ (23.00 \pm 0.32)	+ (24.32 \pm 0.27)	+ (22.63 \pm 0.33)
SE22	-	-	-	+ (23.66 \pm 0.37)
	1.39 * 10 ⁴	+ (20.95 \pm 0.46)	+ (22.44 \pm 0.49)	+ (22.73 \pm 0.17)
	4	+ (23.59 \pm 0.28)	+ (24.90 \pm 0.29)	+ (22.31 \pm 1.05)
CDC_2010K_1441	-	-	-	+ (23.40 \pm 0.49)
	1.52 * 10 ⁴	+ (22.64 \pm 0.24)	+ (23.97 \pm 0.28)	+ (23.45 \pm 0.90)
	5	+ (22.07 \pm 0.80)	+ (23.27 \pm 0.79)	+ (22.56 \pm 0.02)

Cq – Cycle quantification threshold, where the fluorescent is higher than the background.
+ = *Salmonella* positive by the method. In the case of IAC stands for positive signal for IAC.
- = *Salmonella* negative by the method.

Table 7. *Salmonella* Enteritidis (SE) detection by *prot6E/invA* multiplex qPCR and USDA culture method in pooled eggs artificially contaminated. Cq values are given in parentheses.

4. Discussion

This study reports the development of a multiplex qPCR TaqMan assay that allowed for the fast and accurate detection of SE cells from eggs. The assay performed comparably to the traditional SE culture methods described in Chapter MLG 4.05 (USDA) for the detection of SE from meat, poultry, pasteurized egg and catfish products. The overall analysis took roughly 24 h, in contrast to the 5 days to 2 weeks that traditional microbiological culture methods often take. It is noteworthy that agreement between the qPCR and the two microbial culture methods was 100% for all artificially spiked samples.

This novel quantitative real-time PCR (qPCR) assay uses specific primers for the detection of *prot6E* and *invA* genes of SE with TaqMan probes. This assay also includes an internal amplification control (IAC) to detect potential PCR inhibitors that may be present in egg samples. It has become increasingly evident that there is a need for internal controls for PCR reaction, to rule out the presence of PCR inhibitors that can cause false negative results for *Salmonella*-positive samples (Hartman et al., 2005; Hoorfar et al., 2004). The inclusion of this internal control did not affect either the amplification or the detection limit of the qPCR assay. The qPCR developed here as opposed to an *invA* single target qPCR method (Feder et al., 2001; Malorny et al., 2004; Malorny B et al., 2003; Malorny et al., 2003; Bohaychuk et al., 2007; Gonzalez-Escalona et al., 2009) is able to detect specifically SE strains by the use of an SE specific marker (*prot6E*). Additionally it is capable to detect other SE that might lack the *prot6E* gene (Malorny et al., 2007a), such as the case for SE-10. The lack of *prot6E* in SE strains has been co-related with the absence of the SE virulence plasmid (~ 55 kb) (Malorny et al., 2007a). Due to the importance of that plasmid in SE virulence (Bakshi et al., 2003), without it SE has a diminish virulence, and such could be the case for SE-10 which was isolated from chicken. Moreover this assay has a further advantage in that it is an open formula assay, whereby no primers, no probes or IAC are patented or proprietary.

Among the most common gene targets used for SE detection by qPCR are: 1) *Sdf1*, a chromosomal fragment (Agron et al., 2001); 2) *sefA*, encoding for fimbrial antigen SEF14 (Seo et al., 2004); and 3) *prot6E*, encoding for a unique surface fimbriae (Malorny et al., 2007a; Clavijo et al., 2006). *Sdf1* is highly specific for SE but is missing in SE phage types (PT) 6A, 9A, 11, 16, 20, and 27 and besides that were only tested on pure cultures (Malorny et al., 2007a). *SefA* gene is also present in all members of *S. enterica* serogroup D (Gallinarum, Pollorum, Dublin, Rostock, and Typhi, among others) which might lead to false positives results (Seo et al., 2004; Malorny et al., 2007a) and therefore it is not recommended for specific identification of SE. *prot6E* is present in the SE 60 kb virulence plasmid, which is present in most SE (>90%) (Chu et al., 1999; Helmuth and Schroeter, 1994; Clavijo et al., 2006) and therefore was our target of choice for SE specific detection by qPCR.

The detection limit of this qPCR assay was ~ 40 copies of genomic DNA. Usually 1 ml of pre-enrichment is boiled and 2 µl of supernatant is used for qPCR reaction. Thus, a population of approximately 4×10^4 CFU/ml needs to be reached in the pre-enrichment to render a positive result. Commonly SE levels reach ~ 10^8 CFU/ml in the pre-enriched cultures. Therefore, this assay could be used for identification and/or quantification of SE cells in foods directly after pre-enrichment. It is also important to note, however, that in non-host environments, *Salmonella* persists most likely in a starved and highly stressed state. However, the addition of a requisite pre-enrichment step in culture media substantially increases cell number. Thus, a pre-enrichment culture provides an essential preliminary step in the application of this assay to the reliable detection of SE from eggs.

Rather than performing replicates of several inoculations with the same strain, we opted to spike the eggs in Table 7, with 9 different SE strains. This provided, in our opinion, a more powerful approach than simply repeating the experiment with the same strain multiple times as other investigators usually do. The ultimate goal of the assay is to detect different SE strains. Thus increasing the bio-complexity of testing provided a more thorough and rigorous challenge to the capability of the qPCR method to detect SE, in general. The IAC amplification was not affected in all the samples tested, however a possible failure in samples containing high levels of SE could be expected. That sort of possible failure is not un-expected given the competitive nature of this qPCR reaction, where primers and probes for *Salmonella* two targets are in excess. Thereby favoring SE targets instead of the DNA IAC. Nevertheless, it is important to emphasize that performance of the IAC in the presence of low DNA copy numbers or in the observed absence of SE was robust and reliable for each food sample analyzed, an imperative finding for any *Salmonella* detection qPCR assay (Malorny et al., 2007b).

In addition to being both effortless and reproducible, the use of ready to use mixtures, such as the one used in this study, facilitate performance of the assay. Likewise, conventional PCR methods are incapable of producing products with known identity (*i.e.* DNA sequence), subsequently failing to ensure proper specificity of PCR product(s) (Rahn et al., 1992; Malorny B et al., 2003; Malorny et al., 2003). Additionally, we employed TaqMan probes for our qPCR assay which had several advantages over the use of non-specific (although cheaper) SYBR Green I assays, including greater sensitivity and a probe-based sequence-specific verification of PCR product identity (Wittwer et al., 1997; Fey et al., 2004; Jacobsen and Holben, 2007).

In conclusion, we have developed a method that has the potential to be used as an initial screen for pre-enrichment cultures for SE without precluding the USDA culture method which is deemed necessary to yield a physical isolate that is acceptable to the regulatory process. This assay showed a high selectivity, accuracy and detection capacity. In addition, we believe that this assay will reduce the amount of samples, overall time, and effort expended in the laboratory since only positive samples will be further processed after the initial pre-enrichment step. As an added benefit, this is also a quantitative assay which allows for SE quantification in pre-enrichments or other samples. Last but certainly not least, the inclusion of the IAC makes it useful for rapid diagnosis of SE in foods directly. Moreover, in order to be applied extensively, collaborative studies should be conducted to assess the inter-laboratory reproducibility of this assay.

5. Acknowledgement

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6. References

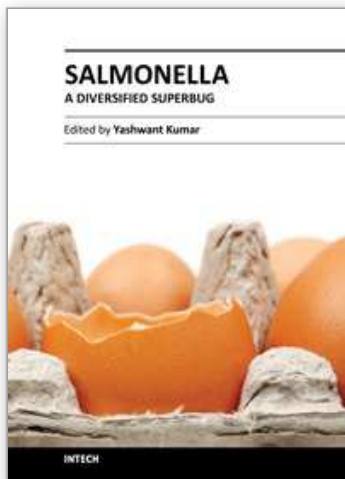
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Salmonella - A Diversified Superbug

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Salmonella is an extremely diversified genus, infecting a range of hosts, and comprised of two species: enterica and bongori. This group is made up of 2579 serovars, making it versatile and fascinating for researchers drawing their attention towards different properties of this microorganism. Salmonella related diseases are a major problem in developed and developing countries resulting in economic losses, as well as problems of zoonoses and food borne illness. Moreover, the emergence of an ever increasing problem of antimicrobial resistance in salmonella makes it prudent to unveil different mechanisms involved. This book is the outcome of a collaboration between various researchers from all over the world. The recent advancements in the field of salmonella research are compiled and presented.

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