Novel *Salmonella* spp. Diagnostic Markers Based on the *gyrB* Gene and its Application on Food and Environmentally-Derived Water Samples

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Abstract: Salmonellosis is a major cause of gastrointestinal infection that generally occurs through the ingestion of fecally contaminated food or water. Molecular markers based on the *gyrase B* (*gyrB)* gene sequence, a Type II DNA topoisomerase subunit enzyme family member, were developed to specifically and sensitively discriminate *Salmonella* spp. from closely related and collocated microorganisms in a water environment. For this, *gyrB* gene sequences of *Salmonella* spp., *E. coli*, *Yersinia enterocolitica*, *Klebsiella pneumoniae* and *Shigella* spp. were aligned and found up to 88% similar. Markers amplified from primers specific to the *Salmonella gyrB* sequences were conserved across 13 *S. enterica* and one *S. bongori* serovar, and were able to detect approximately one *S.* Enteritidis genome. These were more specific and sensitive than the international standard *invA* gene-based *Salmonella* marker. The *gyrB* markers detected 725 *Salmonella* genomes in 100 mL of seeded environmental water sediment sample and 72 *Salmonella* genomes in 300 g of seeded minced samples. Successful detection of *Salmonella* in non-inoculated minced samples was also achieved, with higher sensitivity than the *invA* markers. These makers should be useful in future risk analyses and standards setting for *Salmonella* presence in food, and water used for irrigation and recreational purposes.

Keywords: *gyrB* gene, pathogen detection, PCR, *Salmonella*, water, food.

INTRODUCTION

The *Salmonella enterica* family comprises a diverse range of *Salmonella* spp. including approximately 2557 known serotypes that may cause zoonotic diseases in humans and animals [1]. Salmonellae often associated with faecally contaminated food or water is responsible for approximately 600 deaths per annum in the USA, indicating that the disease is a major health concern [2]. Approximately 1200 cases of Salmonellosis are reported per annum in Australia [3]. Although most of the reported cases were foodborne, less than 1% of gastroenteritis outbreaks in Australia are classified as waterborne or suspected waterborne *via* contaminated drinking and recreational water [4]. More than half of the waterborne-related outbreaks are caused by *Salmonella* spp. and the remaining are caused by other foodborne and waterborne parasites, viruses and bacteria [5].

Rapid, sensitive and specific methods are required to identify and monitor *Salmonella* and safeguard the public from foodborne and waterborne-related outbreaks. For this, molecular techniques offer advantages of speed and accuracy over the traditional culture, microscopy or standard biochemical techniques [6-8]. These methods also overcome issues with under-representative detection of viable but non-culturable cells (VBNC) [9-11]. In particular, sequence-specific polymerase chain reaction (PCR)-based methods are increasingly being used for fast, sensitive and accurate detection and diagnosis of pathogen contaminants [6, 12].

Several gene sequences have been targeted as putative diagnostic markers to specifically detect *Salmonella* spp. including; the *oriC*, *ompC* and *invA* genes [13-15]. However, primers designed to amplify *oriC* and *ompC* sequences produced non-*Salmonella*-specific amplicons from related organisms such as *Citrobacter* spp., *Yersinia enterocolitica, Klesbsiella oxytocanm, Proteus vulgaris* and *Hafina alvei* [16]. The *invA* gene is widely used as an international standard to detect *Salmonella* in both food [17] and environmental samples [18, 19]. A PCR assay based on the *invA* gene was able to detect as few as 10 copies of purified *Salmonella* genomic DNA with the inclusion of 300 copies of an Internal Amplification Control (IAC) [16]. The sensitivity of the same *invA* gene marker was also observed to be comparable to that developed by Malorny *et al.* 2003 [16] when assessing one millilitre of *Salmonella*-seeded homogenate food sample [17]. However, it was also noted that PCR products of the same approximate size were also amplified from several *E. coli* strains [16].

As an alternative to previously targeted sequences, the *gyrB* gene sequence may be sufficiently polymorphic to differentiate *Salmonella* spp., in particular *S. enterica*, from closely related

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microorganisms such as *E. coli* and *Shigella* spp. [20-22]. The *gyrB* gene is a member of the Type II DNA topoisomerase subunit enzyme family [23], which catalyse breakage and reformation of double stranded DNA [24]. Although present in all organisms, the *gyrB* gene sequence differs among microorganisms at the sub-species level, reportedly due to greater evolutionary divergence in comparison to ribosomal gene sequences [20, 25-27]. For example, *E. coli* and *Shigella sonnei* were 0.2% different within the 16S rRNA sequence but 1.9% different within the *gyrB* sequence [20, 24, 28]. The average difference in *gyrB* sequence between *Salmonella* spp. and *E. coli* and *Shigella* spp. is 9.4% and 9.3%, respectively [20] and from other related genera such as *Yersinia* and *Klebsiella* is 22.9% and 10.8%, respectively [20].

Therefore, the aims of this study were to: (1) develop a novel *gyrB* gene PCR-based marker to differentiate *Salmonella* spp. from closely related microorganisms that are commonly found in environmental water and food samples; (2) determine the ability of the *gyrB* marker to detect *Salmonella* spp. in seeded chicken mince and environmentally-derived water sediment samples; and (3) compare the specificity and sensitivity of the *gyrB* marker with the *invA* marker, currently used as the international *Salmonella* diagnostic gene standard.

MATERIALS AND METHODS

Bacterial Culture Strains and Genomic DNA Isolation

Clinical isolates of 13 different serovars of *Salmonella enterica*, one serovar of *Salmonella bongori*, six *Escherichia coli* strains, two *Shigella* spp. strains and an isolate each of *Campylobacter jejuni* and *Bacteroides fragilis* were obtained from either the Department of Microbiology and Immunology, Microbial Diagnostic Unit of The University of Melbourne, Victoria, Australia; Micromon at Monash University,

Table 1: Clinical Bacterial Strains Included for Assessment of Specificity of Novel *gyrB* Markers

Microorganism	Strain/Serovar	Source
Escherichia coli	ATCC 259922	MDU
	OIII:K58	Micromon
	1848	Micromon
	DH5:2	Micromon
	K12	Micromon
	TGI	Micromon
Shigella	*flexneri*	Micromon
	boydii	Micromon
Campylobacter jejuni	NCTC 11392	MDU
Bacteroides	*Fragilis* NCTC 9343	MDU
Salmonella enterica	Enteritidis	MDU
	Enteritidis	IMVS
	Typhimurium	IMVS
	Adelaide	IMVS
	Dublin	IMVS
	Heidelberg	IMVS
	Paratyphi B var Java	IMVS
	Newport	IMVS
	Saintpaul	IMVS
	Infantis	IMVS
	Derby	IMVS
	Brandenburg	IMVS
	Bredeney	IMVS
	IIIb 61:i:z_{53}	IMVS
Salmonella	Bongori (66:z_{65}:-)	IMVS

Where: MDU = Microbial Diagnostic Unit, The University of Melbourne, Victoria, Australia; Micromon = Monash University, Victoria, Australia; and IMVS = Institute of Medical and Veterinary Science, Adelaide, South Australia, Australia.

Victoria, Australia or the Institute of Medical and Veterinary Science, Adelaide, South Australia, Australia (Table **1**).

Salmonella spp., *E. coli* and *Shigella* spp. were grown on nutrient agar (Oxoid, Australia) at 37 °C for 24 h. Single colonies were grown on XLD selective medium and MacConkey agar (Oxoid, Australia). After identification using Microbact^{TM} 24E (Oxoid, Australia), a single colony was picked and inoculated into 10 mL of nutrient broth and grown at 37 °C for 24 h. *Campylobacter jejuni* and *Bacteriodes fragilis* were anaerobically grown on *Campylobacter* selective media (Oxoid, Australia) at 47 °C and *Bacteroides* Bile Esculin Agar (Oxoid, Australia) at 37 °C for 48 h, respectively. Single colonies were resuspended directly into 1.5 mL of nutrient broth. The inoculated nutrient broth was then centrifuged at 14,000 *g* for 2 min to obtain a cell pellet. The supernatant was discarded and total genomic (g)DNA was extracted with the Ultraclean Microbial DNA Isolation Kit (MoBio, Australia). DNA quality and quantity were assessed by agarose gel electrophoresis.

A Novel *gyrB* Marker for *Salmonella* spp.: Specificity and Sensitivity

Initially, a set of 81 *gyrB* sequences (~1200 bp) were accessed from the GenBank databases (www.ncbi.nim.nih.gov) comprising 38 strains of *E. coli*, 15 strains of *Shigella flexneri*, 12 strains of *Shigella sonnei*, 1 strain of *Klebsiella pneumonia*, 1 strain of *Yersinia enterocolitica* and 14 strains of *Salmonella* spp. The strain-specific sequences from within each bacterial species were then aligned using ClustalW (www.ebi.ac.uk/Tools/ clustalw) to provide a consensus *gyrB* sequence for each species. Subsequently, the species consensus *gyrB* sequences were aligned to identify *Salmonella*-specific sequence suitable for development of novel and discriminatory PCR primers. The *Salmonella*-specific primer sequences were 100% homologous to all of the *Salmonella* strains assessed.

The *gyrB* gene primers were assessed in 25 µL PCR reactions comprising PCR buffer (Scientifix, Australia), 1.5 mM $MgCl_2$ (Scientifix, Australia), 200 µM of each dNTP (Scientifix, Australia), 0.4 µM of each primer (Sigma Genosys, Australia), 1.25 units of *Taq* Polymerase (Scientifix, Australia) and 100 ng of gDNA (Table **1**). The thermal cycling conditions were; initial denaturation at 95 °C for 5 min, followed by 30 cycles of 96 °C for 1 min, 63 °C for 1 min and 72 °C for 1 min with a final extension at 72 °C for 1 min. The *gyrB* marker was compared for specificity against the *invA* gene marker [14, 16] to discriminate the *Salmonella* from the non-*Salmonella* species strains (Table **1**). The PCR reaction and its thermal cycling conditions for the amplification of the *invA* marker, using the primer pair 139F and 141R, were as previously described [14, 16]. Markers were visualised on agarose gel, excised, purified using Freeze N' Squeeze DNA Gel Extraction Kit (Bio-Rad, Australia), cycle sequenced by Macrogen, Korea (www.macrogen.com) and BLASTN searched for similar sequences against the NCBI GenBank databases.

Sensitivity of the *gyrB* marker was assessed using a 10-fold dilution series of 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg or 10 fg of *S. enterica* serovar Enteritidis gDNA extracted from pure culture. Traditional PCR conditions were as previously described and genome copy number was calculated based on the molecular weight of *S. enterica* serovar Enteritidis (~5.0 fg/genome; www.sanger.ac.uk/Projects/Salmonella/) where genome copy number = (concentration of sample per µL / weight of 1 genome in ng) X gene copies per genome. In general, 2.5 pg *Salmonella* genomic DNA is approximately equivalent to 500 genomes [29].

Quantitative Real-Time (qRT) PCR assays were performed on an IQ5 thermocycler (Bio-Rad, Australia) with the *Salmonella*-specific *gyrB* primers in 25 µL volumes comprising 10-fold dilution of pure *S. enterica* serovar Enteritidis gDNA (14.5 ng to 14.5 fg) and *S. enterica* serovar Typhimurium (52.1 ng to 52.1 fg), IQ SYBR Green Supermix (Bio-Rad, Australia) and 0.4 µM of each *gyrB* primer (Sigma Genosys, Australia). Cycle conditions were: Initial denaturation at 95 °C for 1 min; 40 cycles of 95 °C for 10 s, 65 °C for 15 s, 72 °C for 30 s and 83 °C for 10 s (fluorescence reading), followed by melt curve analysis from 65 to 95 °C with 0.5 °C/10 s increments. All reactions were performed in duplicate and mean C_t values were used to calculate the number of gene copies utilising a threshold line of 75. The number of *Salmonella* spp. genome copies in correlation to the DNA concentration (ng/ µL) was calculated using the regression line produced by the standard curve followed by the calculation described above, based on the molecular weight of the *Salmonella* genome. The mathematical model of the regression line was equivalent to Y = mX + c, where Y is the C_t value, m is the slope, X is the log of the gene copy number and c is the interception value when Y=0. PCR efficiencies were calculated from the standard

curves using the following equation: PCR efficiency = $[10^{(-1/m)} -1]$ [30].

Detection of *Salmonella* spp. in Environmentally-Derived Water Samples

Water samples (10 L) were collected from a large rural water catchment system in SE Australia between August 2006 and October 2007 [31]. After collection and transportation to the laboratory, 100 mL of each sample was immediately filtered through a 0.45 µM pore size filter membrane (Millipore, Australia) using a vacuum pump. The collected particulate biomass was detached from the filter membrane using a sterile pipette tip and resuspended in 1 mL of sterile ultrapure water. The biomass was then harvested by centrifugation at 18,000 *g* for 2.5 min. The supernatant was discarded and gDNA from the collected pellet was extracted using the Ultraclean™ Soil DNA Isolation Kit (MoBio, Australia). The gDNA was resuspended in 20 µL of sterile water and checked for integrity *via* agarose gel electrophoresis.

In order to demonstrate that the *gyrB* marker was amplifiable from an environmentally-derived water sample background, gDNA from sediment samples previously identified as negative for *Salmonella* using traditional microbiology techniques [32] were seeded with *S.* Enteritidis gDNA. For this, total gDNA from sediment samples was resuspended in 100 µL of sterile H_2O and *S.* Enteritidis gDNA was added to produce a final concentration from 1.45 ng μL^{-1} to 145 fg μL^{-1}. Subsequently, 1 µL of each concentration was used as template in the same qRT-PCR conditions as previously described with the SalEF3/R3 primers to quantify production of the 205 bp marker.

To validate that the *gyrB* marker was able to amplify *Salmonella* from a naturally infected sample, an environmentally-derived water sample (Site 020 March 2007) identified as positive for *Salmonella* using traditional microbiology techniques [32], was used. For this, the previously described traditional PCR conditions were used together with water sediment-derived total gDNA template amounts ranging from 100 ng to 400 ng. The PCR product was visualised on agarose gel, sequenced as previously described and compared with sequences on the GenBank databases to confirm the diagnosis of *Salmonella* spp.

Detection of *Salmonella* spp. in Minced Chicken

Applicability of the *gyrB* markers to detect *Salmonella* spp. in food-derived sample was tested on both artificially and naturally contaminated chicken mince. Both types of experiments were done in triplicate to ensure reproducible and reliable results. Twelve samples of 300 grams of chicken mince were purchased from six different local markets and one major supermarket chain in Australia. Samples were de-identified upon arrival and stored at 4 °C until further processing.

For artificially inoculated samples, 50 grams of the minced samples derived from the local markets were autoclaved and then added with 450 mL of 0.1% buffered peptone water (BPW) (Oxoid, Australia) before being homogenized in a stomacher for 2 min. The homogenized sample was then artificially inoculated with 100 µL of serial diluted BPW containing 10-100, 100-1000, 1000-10000 CFU of *S.* Typhimurium which was previously enumerated for its CFU count on nutrient agar. In relation to food borne illnesses and the ease of comparison between traditional microbiological and molecular based techniques, CFU counts instead of cell numbers were used to inoculate minced chicken samples. Contaminated samples were then pre-enriched by incubating them at 37 °C for 18 hours without shaking as optimised by Trevanich *et al*. 2010 [33]. One mL of each pre-enriched samples containing serially diluted CFU counts was then taken for viable count and gDNA extraction. Genomic DNA was extracted by thermal cell lysis using Chelex100 (Biorad, Australia) according to Malorny *et al.* 2003 [16].

For detection in naturally contaminated samples, 50 g of minced chicken sample from a local market or supermarket was added to 450 mL of BPW and directly homogenized, pre-enriched and extracted as previously described. Extracted gDNA was then amplified with qRT-PCR according to the method described previously. The quantity of the PCR products obtained was plotted against the standard curve to estimate the amount of *Salmonella* contamination in the samples.

For a comparison on how sensitive the *gyrB* marker is with the existing universal gene marker, *invA*, detection of *Salmonella* in minced samples with *invA* marker was also done. Four µL of the extracted gDNA from artificially inoculated and naturally infected samples was used as a template with the addition of IQ SYBR Green Supermix (Bio-Rad, Australia) and 0.2 µM of each *invA* primer (Sigma Genosys, Australia) in a 25 reaction. Cycle conditions were: Initial denaturation at 95 °C for 1 min; 40 cycles of 95 °C for 10 s, 65 °C for 15 s, 72 °C for 30 s and 83 °C for 10 s

(fluorescence reading), followed by melt curve analysis from 65 to 95 °C with 0.5 °C/10 s increments.

RESULTS

A Novel *Salmonella* spp. *gyrB* Gene Marker: Specificity and Sensitivity

Alignment of the consensus *gyrB* sequences revealed a sequence identity of 86% between *Salmonella* spp. and *E. coli*, of 78% between *Salmonella* and *Yersinia enterocolitica* and of 88% equally between *Salmonella* spp. and *Shigella* spp. and *Klebsiella pneumoniae*. The following primer sites were identified as unique to *Salmonella* spp.: SalEF1 5'-ACGCTCTGTCGCAAAAACTG-3' at position 56 to 76 and SalER1 5'-GTGACGGCCAGGGGTGCC-3' at position 138 to 156. Two alternative unique primer sites were also identified: SalEF3 5'-CGTGGGCGTCTC GGTAGTY-3' at position 35 to 54 and SalER3 5'-CTCATATTCAAATTCAGTGACG-3' at position 218 to 241 within *S. enterica* strain SSM1592 *gyrB* (accession DQ386877).

When assessed for specificity against isolates in Table **1**, the SalEF1/R1 and SalEF3/R3 primers amplified a clear and reproducible 100 bp and 205 bp products, respectively from only the *S. enterica* and *S. bongori* samples. Also, the *gyrB* marker sequences amplified among all of the assessed *S. enterica* and *S. bongori* serovars were 100% homologous. In comparison, following the described PCR conditions [28], the 284 bp *inv*A marker was amplified from *E. coli* ATCC 259922, *C. jejuni* NCTC 11392 and *Bacteroides fragilis* NTCC 9343 as well as the *Salmonella* spp.

Following traditional PCR with the SalEF1/R1 and SalEF3/R3 primers, the 100 bp and 205 bp *gyrB* markers were visualised on agarose gel after amplification from just 10 pg of pure *S.* Enteritidis MDU gDNA (Table **1**). This was calculated to be representative of 2000 genomes given an approximate molecular weight of *S. enterica* at 5.0 fg/genome and an assumption of one *gyrB* gene per genome.

The detection sensitivity of *gyrB* gene marker, SalEF3/R3 was tested with quantitative RT-PCR using serially diluted gDNA from the same *S.* Enteritidis and *S.* Typhimurium cultures. A single peak was observed in the post-amplification dissociation curves at 85.50°C. The standard curve for *S*. Enteritidis and *S*. Typhimurium using SalEF3/R3 primers produced R^2 values of 0.996 and 0.997 with slopes of -2.886 and -3.0768, respectively. Both reactions achieved an amplification efficiency of ≥95% with detection sensitivities of 145 fg for pure *S*. Enteritidis and 521 fg DNA for *S*. Typhimurium. These equated to the detection of approximately six *S*. Enteritidis and one *S*. Typhimurium genomes under the same assumptions previously stated (Tables **2** and **3**).

Table 2: Detection and Quantification of Serial Diluted gDNA *Salmonella enterica* Serovar Enteritidis with *gyrB* Marker

S. Enteritidis gDNA (ng/ µL)	Mean C_t	Genome copy numbers (per assay)
14.5	20.57	7.41×10^5
14.5×10^{-1}	23.63	6.45×10^4
14.5×10^{-2}	26.12	8.85×10^3
14.5×10^{-3}	28.53	1.29×10^3
14.5×10^{-4}	32.05	7.80×10^1
14.5×10^{-5}	35.24	6.13×10^0

Table 3: Detection and Quantification of Serial Diluted gDNA *Salmonella enterica* Serovar Typhimurium with *gyrB* Marker

S. Typhimurium gDNA (ng/ µL)	Mean C_t ± SE	Genome copy numbers (per assay)
52.1×10^{-1}	14.10 ± 0.08	8.3017×10^4
52.1×10^{-2}	16.87 ± 0.05	1.05×10^4
52.1×10^{-3}	19.92 ± 0.02	1.07×10^3
52.1×10^{-4}	23.18 ± 0.08	9.3×10^1
52.1×10^{-5}	26.63 ± 0.06	7×10^0
52.1×10^{-6}	29.82 ± 1.38	1×10^0

Ability of *gyrB* Marker to Detect *Salmonella* spp. in Environmentally-Derived Water Samples and Chicken Mince

Following seeding of water-derived sediment samples, the minimum amount of pure *S.* Enteritidis gDNA that was detected using qRT-PCR was 145 fg using the SalEF3/R3 primers. This equated to 725 genomes (per 100 mL of sample).

A clear and reproducible 205 bp marker was amplified from 400 ng (approximately 2.0×10^{-8} genomes per 100 mL of non-seeded sediment-derived gDNA), previously shown to contain *Salmonella*, using the SalEF3/R3 primer pair. The 205 bp marker sequence was highly homologous to published *gyrB* sequence from *S. enterica* and *S. bongori* (e.g. 99% similar to AM933173).

Table 4: Detection of *Salmonella enterica* Serovar Typhimurium from Artificially Contaminated Chicken Mince with *gyrB* Marker

Amount of *S.* Typhimurium (CFU/mL) seeded into samples	Mean C_t ± SE	Genome copy numbers (per 300g)
Negative control (Blank)	35.67 ± 0.37	0
1.0×10^{-6}	17.87 ± 0.42	1.24×10^{6}
1.0×10^{-5}	21.86 ± 0.18	6.25×10^{4}
1.0×10^{-4}	24.79 ± 0.21	6.96×10^{3}
1.0×10^{-3}	28.77 ± 0.21	3.55×10^{2}
1.0×10^{-2}	30.90 ± 0.93	7.2×10^{1}

The SalEF3/R3 marker was also successful in amplifying a reproducible and clear 205 bp band from *Salmonella* in the presence of background microflora and inhibitors in minced chicken. In artificially inoculated samples, *gyrB* marker was able to consistently amplify at least 10^2 CFU/ mL of *S.* Typhimurium (Table **4**). When the average C_t value of the 10^2 CFU/ mL of *Salmonella* in the seeded sample was compared to the standard curve of the pure gDNA of *S.* Typhimurium, the detection limit of 10^2 CFU/ mL equated to approximately 72 genomes per 300 grams of sample.

In non-seeded chicken mince, *Salmonella* was detected from three of the six samples derived from six different local markets. The amplification results were reproducible for each replication with SD values ranging from 0.07 to 0.67. The average C_t values obtained for the three samples were 29.94, 26.16 and 25.99. When the C_t values of the unknown samples were compared against the *S.* Typhimurium standard curve, approximately 148, 2503 and 2843 genomes of *Salmonella* per 300 gram of samples were detected, respectively. Amplification of *Salmonella* in non-seeded food samples indicated that the marker is robust enough to detect *Salmonella* in the presence of background microflora and other inhibitory food components.

Amplification of *Salmonella* spp. in Pure Culture and Chicken Mince with *invA* Marker

Quantitative RT-PCR using serial diluted gDNA from the same *S.* Typhimurium culture with the *invA* primers produced a single peak and an R^2 value of 0.994 with a slope of -3.373. The reaction achieved an amplification efficiency of 107.5% with detection sensitivity of 499.2 fg DNA for *S*. Typhimurium. This equated to the detection of approximately five *S*. Typhimurium (Table **5**) genomes.

The *InvA* gene was able to amplify *Salmonella* from artificially inoculated chicken mince but none of the naturally infected samples. The threshold detection sensitivity of the *invA* in artificially inoculated samples was a minimum of 10^3 CFU/mL of *S.* Typhimurium (Table **6**). This equated to the detection of 832 genomes per 300 grams of sample.

DISCUSSION

Ubiquitous to the environment, *Salmonella* is known as one of the leading causes of food poisoning, accounting for one of the highest morbidity and mortality rates of foodborne diseases worldwide [34]. More than 50% of outbreaks caused by bacteria are attributed to *Salmonella* with *S.* Enteritidis and *S.*

Table 5: Detection and Quantification of Serial Diluted gDNA *Salmonella enterica* Serovar Typhimurium with *invA* Marker

S. Typhimurium gDNA (ng/ µL)	Mean C_t ± SE	Genome copy numbers (per assay)
49.9×10^{-1}	13.25 ± 0.47	6.85×10^{5}
49.9×10^{-2}	16.36 ± 0.18	8.17×10^{4}
49.9×10^{-3}	19.83 ± 0.20	7.67×10^{3}
49.9×10^{-4}	22.93 ± 0.19	9.24×10^{2}
49.9×10^{-5}	25.74 ± 0.11	1.35×10^{2}
49.9×10^{-6}	30.61 ± 0.06	5×10^{0}

Table 6: Detection of *Salmonella enterica* serovar Typhimurium from Artificially Contaminated Chicken Mince with *invA* Marker

Amount of *S.* Typhimurium (CFU/mL) seeded into samples	Mean C_t ± SE	Genome copy numbers (per 300g)
Negative control (Blank)	35.59 ± 1.37	0
1.0×10^{-6}	17.19 ± 0.04	2.32×10^{6}
1.0×10^{-5}	22.57 ± 0.40	5.89×10^{4}
1.0×10^{-4}	25.45 ± 0.20	8.25×10^{3}
1.0×10^{-3}	28.81 ± 0.63	8.32×10^{2}

Typhimurium being reported as the two most prevalent of the known 2500 serotypes [35, 36]. Due to high economic and social impacts caused by salmonellosis, rapid detection and diagnostic methods are imperative to prevent, control and limit infections. The aim of this research was to develop a rapid test for the detection of *Salmonella* on environmentally derived water samples and food samples using traditional and real-time PCR assay, targeting the *gyrB* gene.

National standards for acceptable *Salmonella* dosages are lacking in Australia. There are international and national standards for 'drinking' water quality, however Australian standards governing 'environmental' water quality do not exist. This includes water used to irrigate food crops and for recreational use. Meanwhile, there is a zero tolerance level of *Salmonella* dosage in food based on the 1995 Microbiological Reference Criteria for Food, irrespective of strain [37]. The lack of clarity around microbiological standards is likely due to direct detection and enumeration difficulty in water and food products. This is particularly true for *Salmonella* spp. which may be inaccurately predicated through fecal coliform or *E. coli.* detection [38]. Also, no specific media is able to grow *Salmonella* in isolation, without cultivating other microorganisms such as *Proteus* spp. or even *Pseudomonas* spp. [39, 40].

To enable rapid and specific *Salmonella* spp. detection and direct enumeration from water and food samples, quantifiable *gyrB* gene diagnostic markers were developed. These discriminated *Salmonella* spp. from other water and food-borne fecal contaminants such as *Bacteroides fragilis* and other closely related microorganisms, such as *E. coli* and *Shigella*. They successfully amplified 13 different *S. enterica* serovars as well as *S. bongori*, reflecting a high conservation of the *gyrB* gene within the *Salmonella* genus. Conversely, a suitable rate of evolution was detected within the *gyrB* sequence to discriminate between genera [20]. In comparison, the *invA* marker [14] is not specific to *Salmonella* and amplifies from *E. coli* [16], *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Citrobacter freundii* [14, 41], and *Campylobacter jejuni* and *Bacteroides fragilis* (this study). Cross-amplification with closely related species with a high probable presence in a water environment substantially reduces the diagnostic power of the marker.

Quantitative amplification of the 205 bp *gyrB* marker detected 10 pg (2,000 genomes) of extracted *S. enterica* gDNA while the detection limit of the *invA* gene marker was reported to be 300 cells (genomes); based on the detection of 27 pg of extracted gDNA [14]. However, re-calculation of the *invA* genome copy number (based on 27 pg and the equation described previously) indicated a detection threshold of 5,000 genomes rather than the claimed 300. In seeded water samples, the 205 bp *gyrB* marker detected 725 genomes per 100 ml of seeded environmentally-derived water sample. In non-seeded and naturally infected water samples, a minimum of 400 ng (2.0×10^{-8} genomes per 100 ml) of sediment-derived total gDNA was required for *Salmonella* spp. detection. In chicken mince samples, the *gyrB* marker was also proven to be more sensitive, able to detect as few as 72 genomes in artificially contaminated samples in comparison to the threshold of 832 genomes detected with the *invA* marker.

Immunoassay based commercial kits such as Tecra Unique™ (Tecra, Australia), VIDAS *Salmonella* assay (bioMérieux, France) and 1-2 Test for *Salmonella* (Biocontrol Systems, USA) are used to generate presumptive positive and negative tests for *Salmonella* within two days [34]. Whilst highly sensitive, reportedly detecting as little as one to five CFU per 25 g of sample, results must be confirmed with standard culturing methods [42]. Sensitivity of the kits is also affected by background flora potentially resulting in false negatives [43, 44]. Accordingly, molecular

markers may be more reliable, although also suffer from reduced sensitivity related to PCR efficiency. This may result from competition for primer binding sites within a diverse microflora. Although optimisation of PCR conditions, including annealing temperature, was conducted [45], primer specificity may influence the reaction since *gyrB* sequences from other microbes potentially present in the same environment were unavailable for direct comparison to the *Salmonella gyrB* sequence. Also, the efficiency of the PCR assay may have been affected by the DNA extraction method, which may not have removed all PCR inhibitors such as humic substances, complex carbohydrates or other organic compounds such as blood [40, 46, 47]. The Ultraclean™ MoBio Soil DNA extraction kit (MoBio, Australia) according to Behets *et al.* 2007 [48] and Klerks *et al.* 2006 [49], is an efficient water extraction technique for subsequent PCR amplification. However, further DNA purification methods should be investigated. PCR sensitivity may also be improved by including 30 to 300 copies of an Internal Amplification Control in the PCR mastermix [16]. Also as the level of *Salmonella* contamination in food or water samples may be very low, pre-enrichment step may be useful to enhance the detection limit by allowing multiplication of targeted bacterial cell [50, 51]. Pre-enrichment steps before detection with real-time PCR have increased detection sensitivities by 10 to 1000 fold [52].

Rapid methods to detect the presence of *Salmonella* are crucial to safeguard public safety. The development of *gyrB* gene markers provides a rapid and promising technique to quantify bacterial concentration initially present in samples. Increased diagnostic sensitivity and quantitative measurement provided by quantitative PCR methods will enable pathogen dose-response relationships to be determined for risk assessment and future standards setting for *Salmonella* in water environments and food products [53, 54].

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