Comparative Virulotyping of *Salmonella typhi* and *Salmonella enteritidis*

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Abstract Members of *Salmonella enterica* are important foodborne pathogens of significant public health concern worldwide. This study aimed to determine a range of virulence genes among typhoidal (*S. typhi*) and non-typhoidal (*S. enteritidis*) strains isolated from different geographical regions and different years. A total of 87 *S. typhi* and 94 *S. enteritidis* strains were tested for presence of 22 virulence genes by employing multiplex PCR and the genetic relatedness of these strains was further characterized by REP-PCR. In *S. typhi*, *invA*, *prgH*, *sifA*, *spiC*, *sopB*, *iroN*, *sitC*, *misL*, *pipD*, *cdtB*, and *orfL* were present in all the strains, while *sopE*, *agfC*, *agfA*, *sefC*, *mgtC*, and *sefD* were present in 98.8, 97.7, 90.8, 87.4, 87.4 and 17.2 %, of the strains, respectively. No *lpfA*, *lpfC*, *pefA*, *spvB*, or *spvC* was detected. Meanwhile, in *S. enteritidis*, 15 genes, *agfA*, *agfC*, *invA*, *lpfA*, *lpfC*, *sefD*, *prgH*, *spiC*, *sopB*, *sopE*, *iroN*, *sitC*, *misL*, *pipD*, and *orfL* were found in all *S. enteritidis* strains 100 %, followed by *sifA* and *spvC* 98.9 %, *pefA*, *spvB* and *mgtC* 97.8 %, and *sefC* 90.4 %. *cdtB* was absent from all *S. enteritidis* strains tested. REP-PCR subtyped *S. typhi* strains into 18 REP-types and concurred with the virulotyping results in grouping the strains, while in *S. enteritidis*, REP-PCR subtyped the strains into eight profiles and they were poorly distinguishable between human and animal origins. The study showed that *S. typhi* and *S.*

enteritidis contain a range of virulence factors associated with pathogenesis. Virulotyping is a rapid screening method to identify and profile virulence genes in *Salmonella* strains, and improve an understanding of potential risk for human and animal infections.

Keywords REP-PCR · *Salmonella typhi* · *Salmonella enteritidis* · Virulotyping

Introduction

Salmonella spp. are important foodborne pathogens in humans and animals and pose significant public health concerns worldwide [1]. *Salmonella* establish an infection and cause illness through bacterially-expressed virulence factors interacting with host cells. *Salmonella* virulence factors play active roles in a broad range of pathogenic mechanisms, including adhesion, invasion, intracellular survival, systemic infection, fimbrial expression, antibiotic resistance, toxin production, and Mg²⁺ and iron uptake [2, 3]. Many of these virulence factors are located in *Salmonella* pathogenicity islands (SPIs) and on plasmids [4–6]. At least 17 SPIs have been identified in different *Salmonella* serovars [7]. SPI-1 and SPI-2, which encode two separate type three secretion systems, TTSS-1 and TTSS-2, respectively, are the most prominent SPIs. SPI-1 encodes virulence genes responsible for invasion while SPI-2, which is absent in *Salmonella bongori*, encodes genes essential for systemic infection inside the host cells [2, 8]. Virulence plasmids encode genes for systemic disease in non-typhoid *Salmonella* serovars such as *Salmonella typhimurium*, *S. enteritidis*, *Salmonella dublin*, and *Salmonella choleraesuis*. On the other hand, *S. typhi* and

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Salmonella paratyphi A do not harbor any plasmid virulence genes but still capable of causing disease [5].

Two *Salmonella* serovars were chosen for this study, typhoidal (*S. typhi*) and non-typhoidal (*S. enteritidis*). *S. typhi*, a human restricted host, is the etiologic agent of typhoid fever, of which there are estimated 16 million annual cases with 600,000 deaths worldwide [9]. Although, the presence of *S. typhi* in the environment is highly rare, the infection is most often acquired through ingestion of contaminated food or water [10]. *S. typhi* is still a pathogen of concern in developing world, especially Asia [11]. *S. enteritidis* is a broad-host-range non-typhoidal serovar and usually cause gastroenteritis in a wide range of unrelated host species [12]. Therefore, it is very important to understand the pathogenic lifestyle of typhoidal and non-typhoidal *Salmonella* serovars. We employed and developed a multiplex PCR (mPCR) for detection of a set of virulence genes that are associated with adhesion, invasion, intracellular survival, colonization, and systematic infection, and also their locations inside the bacterial genome (pathogenicity islands, plasmids, fimbriae, and pathogenicity islets). mPCR approach is useful to detect several short-sequenced genes of interest in one single reaction. It has been performed by many researchers in laboratories due to its high sensitivity and specificity [13–15].

REP-PCR is a genomic fingerprinting method based on PCR amplification of the repetitive DNA sequences dispersed throughout the bacterial genome [16]. An alternative approach to distinguish *Salmonella* species and subspecies based on absence or presence of virulence genes, termed “virulotyping” has been proposed [1, 17, 18]. This study aimed to determine a range of virulence genes among *S. typhi* and *S. enteritidis* strains by mPCR. REP-PCR was carried out to assess the possible genetic relatedness among the tested strains as it is a rapid and fairly discriminative molecular subtyping tool.

Materials and Methods

A total of 87 *S. typhi* strains (77 clinical, 10 environmental) previously described by Thong et al. [19–21] were tested. These strains were from different geographical regions (Malaysia, Indonesia, Papua New Guinea, and Chile) between years 1983 and 2007, and from different outbreaks and sporadic cases. A total of 94 *S. enteritidis* strains (humans = 32, animals = 62) isolated between 2003 through 2008 from Malaysia were analyzed. The zoonotic strains were isolated from poultry while the clinical strains were isolated from hospitalized patients [22, 23]. All the 181 strains were recovered from glycerol stocks kept at -80°C , revived and purity checked by using selective media XLD agar. The strains were grown on Luria–Bertani agar (LB agar) (Oxoid, England) overnight at 37°C .

Crude genomic DNA prepared for each strain by direct boiled cell lysate. A loopful of bacterial cells was suspended in 100 μl of double-distilled water. The suspension was heated at 99°C for 5 min, chilled in ice, centrifuged at $13,400\times g$ for 1 min, and then a 50 μl of clear supernatant transferred to another microfuge tube and used as a DNA template for subsequent PCR analysis.

Primers for the selected virulence genes were as previously published: *cdtB*, *iroN*, *lpfC*, *pefA*, *prgH*, *sifA*, *sitC*, *sopB*, *spvB* [3], *misL*, *orfL*, *pipD*, *sopE*, *spiC* [24], *agfA*, *sefC*, *sefD* [25], *lpfA* [26], *agfC* [27], *invA* [28], *mgtC* [29] and *spvC* [30]. Initially, a monoplex PCR was performed to determine the specificity of each primer. PCR products were purified using DNA purification kit (iNtRON Biotechnology, Korea) and sequenced to validate their identities. Subsequently, the 22 primer pairs were divided into five mPCR sets to differentiate the different sized amplicons. The mPCR I amplified *prgH*, *sopE*, *sifA*, *spiC*, *sopB*, and *pefA*; mPCR II amplified *iroN*, *sitC*, *misL*, *pipD*, *orfL*, and *invA*; mPCR III amplified *sefC*, *mgtC*, *agfA*; mPCR IV amplified *lpfC*, *agfC*, *cdtB*; and mPCR V amplified *spvB*, *spvC*, *sefD*, *lpfA*. Amplification was performed in a 25 μl reaction mixture that included 5 μl (~ 100 ng) of DNA template, $1\times$ of PCR buffer (Promega Inc., Madison, WI, USA), 3 mM of MgCl_2 , 0.75 U of *Taq* DNA polymerase (Promega Inc., Madison, WI, USA), 400 μM of dNTPs mix (Promega Inc., Madison, WI, USA), and 0.4 μM of each primer. The cycling conditions were: 95°C for 5 min, 30 cycles of 94°C for 30 s, 56.3°C for 30 s, 72°C for 2 min, with a final cycle of 72°C for 10 min. PCR products were separated on 1.5 % (wt/vol) agarose gels, stained with ethidium bromide, visualized and photographed under ultraviolet light in a gel documentation system (Bio-Rad, Molecular Imager Gel DocTM, XR Imaging System).

Genetic relatedness of the strains was determined using REP-PCR (primer 5'-GCG CCG ICA TGC GGC ATT-3') according to Bennasar et al. [31]. The cycling condition consisted of 2 cycles of 94°C for 5 min, 33°C for 5 min, and 68°C for 5 min, followed by 30 cycles of 94°C for 1 min, 45°C for 1 min, and 68°C for 1 min, with a final cycle of 68°C for 16 min. REP-PCR products were separated on 1.5 % (wt/vol) agarose gels. The electrophoresis was run for 5 h at 100 V. Standard DNA markers used were 1 kb and 100 bp ladders (Promega Inc., Madison, WI, USA). Gels were stained with ethidium bromide (0.5 $\mu\text{l}/\text{ml}$) for 5 min and destained for 15 min in ddH_2O , and then visualized and photographed under ultraviolet light in a gel documentation system (Bio-Rad, Molecular Imager Gel DocTM, XR Imaging System).

A cluster analysis of the strains based on the presence and absence of virulence genes and REP-PCR profiles was performed by BioNumerics (version 6.0; Applied Maths, Belgium), and a dendrogram was constructed by

the unweighted pair-group method for arithmetic averages (UPGMA).

Results and Discussion

Initial attempts to follow the published PCR conditions [3] yielded non-specific bands. Hence, the annealing temperature and time were modified to 56.3 °C for 30 s. The number of cycles was increased to 30; the concentrations of each primer, dNTPs and DNA template were doubled. DNA sequence analysis of the representative amplicons produced by each primer pair showed high identity ranging from 95 to 100 % to the available GenBank database (data not shown). Representative PCR amplicons are shown in Figs. 1 and 2.

Eleven of the 22 genes tested (*invA*, *prgH*, *sifA*, *spiC*, *sopB*, *iroN*, *sitC*, *misL*, *pipD*, *cdtB*, and *orfL*) were found in 100 % (87/87) *S. typhi* strains. while 15 of the 22 genes tested (*agfA*, *agfC*, *invA*, *lpfA*, *lpfC*, *sefD*, *prgH*, *spiC*, *sopB*, *sopE*, *iroN*, *sitC*, *misL*, *pipD*, and *orfL*) were also found in 100 % (94/94) *S. enteritidis* strains. In *S. typhi* strains, the *sopE* gene was absent from one Chilean strain (ST VC3131/84). The genes *agfA* and *agfC* were found in 90.8 % (79/87) and 97.7 % (85/87) of strains, respectively. Both *sefC* and *mgtC* were found in 87.3 % (76/87) of strains. The prevalence of gene *sefD* was lower at 17.2 % (15/87). The remaining virulence genes (*lpfA*, *lpfC*, *pefA*, *spvB*, and *spvC*) were absent in all the *S. typhi* strains. Meanwhile, in *S. enteritidis* strains, the *sifA* gene was absent from one strain (SE 5558/03) and *spvC* gene was also absent from one strain (SE 7501/03). The genes *pefA*, *spvB* and *mgtC* were found in 97.8 % (92/94) *S. enteritidis* strains while the gene *sefC* was found in 90.4 % (85/94).

The *cdtB* is the only gene that was absent from all *S. enteritidis* strains.

The *invA* and *prgH* genes belong to SPI-1 while the *sifA* and *spiC* genes belong to SPI-2 and they are associated with TTSSs [2, 3]. *sifA* gene associated with intracellular survival and replication in SCV (*Salmonella*-containing vacuole) [32]. Only one *S. enteritidis* strain did not have *sifA* gene. The *sitC* gene belongs to SPI-2 and is important for iron uptake [3]. Other chromosomal genes, *misL*, *orfL*, and *pipD* are located respectively in SPI-3, SPI-4, and SPI-5 and are also associated with TTSSs [2]. The *sopB* gene is located in SPI-5, associated with TTSS-1, and it is required for full virulence in a murine model [3]. The *sopE* gene which is located in SPI-7 and associated with TTSS-1 was present in all the strains tested except one human *S. typhi* strain (ST VC 3131/84). *sopE* gene may not be needed for full virulence if *sopB* is present [33]. This particular strain, ST VC 3131/84, has the *sopB* gene. The *mgtC* gene is located in SPI-3 and is important for bacterial growth at low Mg^{+2} concentrations inside the host cell [2, 34].

The *cdtB* and *iroN* genes are thought to be important in *Salmonella* virulence [3]. The *iroN* gene is for iron uptake while *cdtB* gene is for toxin production [35, 36]. *cdtB* gene is not only limited to *S. typhi* but it is also found in *S. paratyphi A* [37], *Salmonella bredeney*, *Salmonella brandenburg*, and *Salmonella schwarzengrund* [3]. No *cdtB* gene was detected in any of the *S. enteritidis* strains. The environmental strains of *S. typhi* tested in this study were previously sampled from the sewage-polluted Mapocho River, Santiago, Chile [21]. *S. typhi* from this contaminated water and typhoid patients were closely related as determined by PFGE [21]. In this study, the presence of the *cdtB* gene in all the environmental and the human strains from Chile further supports a possible relationship between the

Fig. 1 A montage of amplification products of virulence genes in *S. typhi* using the optimized multiplex PCR amplification. **a** Multiplex PCR (MP I) amplifying (from top to bottom) *prgH* (657 bp), *sopE* (642 bp), *sifA* (449 bp), *spiC* (301 bp), and *sopB* (220 bp); lane 1, 100 bp ladder; lane 2, *S. typhi* strain. **b** Multiplex PCR (MP II) amplifying (from top to bottom) *iroN* (1205 bp), *sitC* (768 bp), *misL* (561 bp), *pipD* (399 bp), *orfL* (332 bp), and *invA* (289 bp); lane 1,

100 bp ladder; lane 2, *S. typhi* strain. **c** Multiplex PCR (MP III) amplifying (from top to bottom) *sefC* (1,103 bp), *mgtC* (655 bp), and *agfA* (151 bp); lane 1, 100 bp ladder; lane 2, *S. typhi* strain. **d** Multiplex PCR (MP IV) amplifying (from top to bottom) *agfC* (308 bp) and *cdtB* (268 bp); lane 1, 100 bp ladder; lane 2, *S. typhi* strain. **e** Multiplex PCR (MP V) amplifying *sefD* (374 bp); lane 1, 100 bp ladder; lane 2, *S. typhi* strain

Fig. 2 A montage of amplification products of virulence genes in *S. enteritidis* using the optimized multiplex PCR amplification. **a** Multiplex PCR (MP I) amplifying (from top to bottom) *prgH* (657 bp), *sopE* (642 bp), *sifA* (449 bp), *spiC* (301 bp), *sopB* (220 bp), and *pefA* (157 bp); lane 1, 100 bp ladder; lane 2, *S. enteritidis* strain. **b** Multiplex PCR (MP II) amplifying (from top to bottom) *iroN* (1,205 bp), *sitC* (768 bp), *misL* (561 bp), *pipD* (399 bp), *orfL* (332 bp), and *invA* (289 bp); lane 1, 100 bp ladder; lane 2, *S.*

enteritidis strain. **c** Multiplex PCR (MP III) amplifying (from top to bottom) *sefC* (1,103 bp), *mgtC* (655 bp), and *agfA* (151 bp); lane 1, 100 bp ladder; lane 2, *S. enteritidis* strain. **d** Multiplex PCR (MP IV) amplifying (from top to bottom) *lpfC* (641 bp) and *agfC* (308 bp); lane 1, 100 bp ladder; lane 2, *S. enteritidis* strain. **e** Multiplex PCR (MP V) amplifying *spvB* (717 bp), *spvC* (571 bp), *sefD* (374 bp) and *lpfA* (250 bp); lane 1, 100 bp ladder; lane 2, *S. enteritidis* strain

strains from contaminated water and human infection with *S. typhi*.

Fimbrial genes are located in the chromosome and on the plasmid. The *agfA*, *agfC*, *lpfA*, *lpfC*, *sefC*, and *sefD* genes, along with *pefA* gene are important for adherence to different sites of the host cells; any loss of these fimbrial genes will decrease the ability of *Salmonella* for adherence in host cells [8]. Comparative genome analysis showed that *S. enteritidis* shares 12 of 13 fimbrial operons and 8 fimbrial pseudogenes with *S. typhi*. Thus, no *lpfA* and *lpfC* genes were detected in *S. typhi* strains and concurred with previous studies since they showed that *S. enteritidis* *lpf* operon is absent from the sequence of *S. typhi* [38, 39].

The *spvB*, *spvC*, and *pefA* genes are located on plasmids and contribute in adhesion and systemic infection against the host cells [5, 32]. These genes are present in other host adapted *Salmonella* serovars such as *S. dublin*, *Salmonella pullorum*, *Salmonella gallinarum*, *S. choleraesuis*, *S. enteritidis*, and *S. typhimurium* [5, 40], however, not all plasmid-bearing serovars contain these virulence plasmid genes [3, 24]. The three plasmid genes (*spvB*, *spvC*, and *pefA*) were absent in *S. typhi* but present in *S. enteritidis* (except in two zoonotic strains).

The overall results showed that the virulence genes are widely distributed among *S. typhi* and *S. enteritidis* strains, regardless the source of isolation (humans, animals, or environment), geographical regions, or the year of isolation. Both serovars contain a range of virulence genes that can allow them to cause diseases and interact with their host cells. Most of the virulence genes that are associated with SPIs (SPI-1, SPI-2, SPI-3, SPI-4, and SPI-5) and TTSSs, along with one pathogenicity islet gene (*iroN*), are shared between the two serovars. Moreover, the complete

absence of three plasmid genes (*pefA*, *spvB*, *spvC*) and two fimbrial genes (*lpfA*, *lpfC*) from *S. typhi* strains was observed while only *cdtB* gene was completely absent from *S. enteritidis* strains. These differences in virulence factors may contribute to host adaptation and the diversity of *Salmonella* pathogenesis. There are many virulence genes in *Salmonella* genus and some are specific to certain serovars [2, 5, 6]. Some of these virulence genes can be expressed and activated during the term of infection inside the host cells, and other virulence genes are limited or specific to serovars. Recently, the issue of bacterial pathogenesis is now entering a new era of quorum sensing inhibitors. Quorum sensing is a social communication behavior within a population of bacterial cells through small chemical signaling molecules. In infectious diseases, the invading bacteria need to reach a certain cell density to coordinate the expression of virulence genes and overwhelming the host defense mechanisms [41, 42].

S. typhi strains could be subtyped into 12 virulotypes (V1–V12) based on the presence and absence of these 22 virulence genes (Table 1). The majority of the strains ($n = 64$) that had virulotype V1 were from different geographical regions (Malaysia $n = 52/56$, PNG $n = 4/4$, Indonesia $n = 3/3$, and Chile $n = 1/24$). Twelve strains associated with sporadic cases of typhoid fever were also clustered within virulotype V1. The remaining Chilean strains (23/24) were characterized into 9 virulotypes (V3–V8 and V10–V12) (Fig. 3). Two Malaysian strains were grouped within the profile V5, while 2 different Malaysian strains were distinguished into two virulotypes (V2, V9).

The genetic relatedness of the strains was also determined by REP-PCR. REP-PCR subtyped *S. typhi* strains into 18 rep-types (R1–R18) and the majority of the strains

Table 1 Virulotypes among 87 *S. typhi* strains tested

Virulotypes (V)	Gene combinations	No. of Strains
V	<i>agfA-agfC-cdtB-invA-iroN-lpfA-lpfC-mgtC-misL-orfL-pefA-pipD-prgH-sefC-sefD-sifA-sitC-sopB-sopE-spiC-spvB-spvC</i>	
V1	<i>agfA-agfC-cdtB-invA-iroN-mgtC-misL-orfL-pipD-prgH-sefC-sifA-sitC-sopB-sopE-spiC</i>	60
V2	<i>agfA-agfC-cdtB-invA-iroN-misL-orfL-pipD-prgH-sifA-sitC-sopB-sopE-spiC</i>	1
V3	<i>agfA-agfC-cdtB-invA-iroN-misL-orfL-pipD-prgH-sefC-sifA-sitC-sopB-sopE-spiC</i>	1
V4	<i>agfA-agfC-cdtB-invA-iroN-misL-orfL-pipD-prgH-sefC-sefD-sifA-sitC-sopB-sopE-spiC</i>	3
V5	<i>agfA-agfC-cdtB-invA-iroN-mgtC-misL-orfL-pipD-prgH-sefC-sefD-sifA-sitC-sopB-sopE-spiC</i>	11
V6	<i>agfA-cdtB-invA-iroN-misL-orfL-pipD-prgH-sefC-sifA-sitC-sopB-sopE-spiC</i>	1
V7	<i>agfA-cdtB-invA-iroN-mgtC-misL-orfL-pipD-prgH-sifA-sitC-sopB-sopE-spiC</i>	1
V8	<i>agfC-cdtB-invA-iroN-misL-orfL-pipD-prgH-sifA-sitC-sopB-sopE-spiC</i>	4
V9	<i>agfA-agfC-cdtB-invA-iroN-misL-orfL-pipD-prgH-sifA-sitC-sopB-sopE-spiC</i>	1
V10	<i>agfC-cdtB-invA-iroN-mgtC-misL-orfL-pipD-prgH-sifA-sitC-sopB-spiC</i>	1
V11	<i>agfC-cdtB-invA-iroN-mgtC-misL-orfL-pipD-prgH-sifA-sitC-sopB-sopE-spiC</i>	2
V12	<i>agfC-cdtB-invA-iroN-mgtC-misL-orfL-pipD-prgH-sefD-sifA-sitC-sopB-sopE-spiC</i>	1

Fig. 3 Dendrogram generated based on REP-PCR profiles, constructed by UPGMA algorithm (BioNumerics v6.0) showing the relatedness of virulotypes and rep-types of the 87 *S. typhi* strains. R rep-type, V virulotype, H human, E environment, M malaysia,

P papua new guinea, I indonesia, C chile, Y 2007 (2), 2006 (19), 1992 (7), 1991 (4), 1990 (10), 1987 (2), 1986 (11); a black box indicates the presence of virulence gene and a white box indicates of the absence of virulence gene; V1–V12 are profiles of positive virulence gene

belong to rep-type R1 (Malaysia, $n = 50/56$, PNG, $n = 4/4$, and Indonesia, $n = 3/3$) including (11/12) of sporadic strains. Chilean strains (24/24) distinguished into 12 rep-types (R5–R13 and R15–R17). The rep-types (R2–R4 and

R18) belong to Malaysian outbreak strains, while, R14 rep-type belongs to a Malaysian sporadic strain (Fig. 3).

There was a concordance in the virulotyping and REP-PCR typing in the grouping *S. typhi* strains. Both methods

grouped the same majority of strains (Malaysia, Papua New Guinea, and Indonesia) in one profile (V1 and R1), except for the Chilean strains, which were characterized in different profiles. The similarity of virulotypes and rep-types of *S. typhi* strains isolated from Malaysia, Papua New Guinea, and Indonesia indicate the successful clonal spread of this serovar in Southeast Asia region. Based on virulotyping, the differences observed within Chilean strains were mainly due to variation in five virulence genes (*agfA*, *agfC*, *mgfC*, *sefC*, and *sefD*).

In *S. enteritidis*, the strains were subtyped into 6 virulotypes (V1–V6) (Table 2). The majority of the strains belong to V1 ($n = 83$) which was shared by strains isolated from humans, animals, different locations and years. This was followed by V2 ($n = 6$), V3 ($n = 1$), V4 ($n = 2$), V5 ($n = 1$), and V6 ($n = 1$). The virulotype V2 was shared among six animal strains isolated in 2003 and 2005. The virulotypes V3–V6 were associated only with the animals strains isolated in 2003 (Fig. 4).

REP-PCR differentiated the *S. enteritidis* strains to eight rep-types. The two most common rep-types were R1 (humans = 8, animals = 18) and R2 (humans = 13, animals = 11). The other rep-types (R3, R4, R5) were also shared by both human and animal strains except R6 which contains only one human strain, and R7 and R8 comprise only animal strains ($n = 12$ and 6, respectively) (Fig. 4).

Both methods were poorly differentiated *S. enteritidis* strains among different sources, places (within Malaysia only), and years of isolation; these results concurred with previous studies [17, 43]. Thong et al. [22] reported that this serovar lacks the genetic diversity as determined by PFGE due to highly clonal. *S. enteritidis* isolates are endemic in Malaysia and have been genetically quite stable for more than a decade.

Although virulotyping and REP-PCR are fast and simple methods, they lack discrimination and may not be suitable markers for studying genetic relatedness for *S. typhi* and *S. enteritidis*. There is indeed a limitation in this study using

Table 2 Virulotypes among 94 *S. enteritidis* strains tested

Virulotypes (V)	Gene combinations	No. of Strains	
		Humans	Animals
V	<i>agfA-agfC-cdtB-invA-iroN-lpfA-lpfC-mgtC-misL-orfL-pefA-pipD-prgH-sefC-sefD-sifA-sitC-sopB-sopE-spiC-spvB-spvC</i>		
V1	<i>agfA-agfC-invA-iroN-lpfA-lpfC-mgtC-misL-orfL-pefA-pipD-prgH-sefC-sefD-sifA-sitC-sopB-sopE-spiC-spvB-spvC</i>	32	51
V2	<i>agfA-agfC-invA-iroN-lpfA-lpfC-mgtC-misL-orfL-pefA-pipD-prgH-sefC-sefD-sifA-sitC-sopB-sopE-spiC-spvB-spvC</i>	0	6
V3	<i>agfA-agfC-invA-iroN-lpfA-lpfC-mgtC-misL-orfL-pefA-pipD-prgH-sefC-sefD-sifA-sitC-sopB-sopE-spiC-spvB-spvC</i>	0	1
V4	<i>agfA-agfC-invA-iroN-lpfA-lpfC-misL-orfL-pefA-pipD-prgH-sefD-sifA-sitC-sopB-sopE-spiC-spvB-spvC</i>	0	2
V5	<i>agfA-agfC-invA-iroN-lpfA-lpfC-mgtC-misL-orfL-pipD-prgH-sefC-sefD-sifA-sitC-sopB-sopE-spiC-spvC</i>	0	1
V6	<i>agfA-agfC-invA-iroN-lpfA-lpfC-mgtC-misL-orfL-pipD-prgH-sefC-sefD-sifA-sitC-sopB-sopE-spiC</i>	0	1

Fig. 4 Dendrogram generated based on REP-PCR profiles, constructed by UPGMA algorithm (BioNumerics v6.0) showing the relatedness of virulotypes and REP-types of the 94 *S. enteritidis* strains. R rep-type, V virulotype, H human, A animal, Y1 2007 (1),

2006 (10), 2005 (9), 2003 (2); Y2: 2008 (2), 2007 (1), 2006 (2), 2005 (7), 2004 (1), 2003 (10); Y3: 2007 (4), 2006 (2), 2005 (5), 2004 (1), 2003 (2); a black box indicates the presence of virulence gene and a white box indicates of the absence of virulence gene

the current mPCR approach to determine the majority of the virulence genes in both serovars. By using a wider array of virulence genes, a more precise characterization would be possible [17]. However, in a resource-limited situation, such direct and relatively cheap mPCR assay to type the strains based on a repertoire of virulence genes might still be useful.

In conclusion, a mPCR was successfully optimized and developed to detect the selected 22 virulence genes. It served to distinguish typhoidal and non-typhoidal *Salmonella* isolates but not for specific serovar identification. Virulotyping via PCR, when combined with REP-PCR, is a rapid approach to identify and profile virulence genes in typhoidal (*S. typhi*) and non-typhoidal (*S. enteritidis*) strains, and improve our understanding of the potential risk for human and animal infections as additional experiments are needed to fully elucidate it.

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