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Rapid detection and serovar identification of common *Salmonella enterica* serovars in Canada using a new pyrosequencing assay.

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Abstract

Serotyping of *Salmonella enterica* subspecies *enterica* is a critical step for foodborne salmonellosis investigation. We have developed a new assay to identify *Salmonella enterica* subsp. *enterica* (*S. enterica*) serovars based on a triplex polymerase chain reaction (PCR) with pyrosequencing for amplicon confirmation and phylogenetic discrimination of strain. The top 54 most prevalent serovars of *S. enterica* in Canada were examined with a total of 23 single nucleotide polymorphisms (SNPs) and/or variations (SNVs) located on three genes (*fliD*, *sopE2*, and *spaO*). Seven of the most common serovars, including Newport, Typhi, Javiana, Infantis, Thompson, Heidelberg and Enteritidis are successfully distinguished from the other serovars based on their unique SNP/SNV combinations. The remaining serovars, including Typhimurium, ssp I 4,[5],12:i:-, and Saintpaul are further divided into 47 subgroups that demonstrate the relatedness to phylogenetic classifications of each serovar. This pyrosequencing assay is not only cost-effective, rapid, and user-friendly, but also provides phylogenetic information by analysing 23 selected SNPs. With the additional layer of confidence to the PCR results and the accuracy and speed of pyrosequencing, this novel method would benefit the food industry and provides a tool for rapid outbreak investigation through quick detection and identification of common *S. enterica* serovars in Canada.

Keywords

Salmonella enterica, Serotyping, Genotyping, Pyrosequencing, Single Nucleotide Polymorphism (SNP)

Introduction

Salmonella enterica infection is a serious public health concern worldwide. It is estimated about 93.8 million cases of gastroenteritis occur globally each year with 155,000 deaths due to salmonellosis (Majowicz et al. 2010). In Canada, *S. enterica* was the most common cause accounting for approximately 41 % of the reported foodborne outbreaks from 2008 to 2014 (Bélanger P et al. 2015). The species *S. enterica* is currently comprised of 6 subspecies; *S. enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica* (Grimont and Weill 2007). *S. enterica* subsp. *enterica* (*S. enterica*) includes more than 1,500 serovars and accounts for 99 % of all human *Salmonella* infections (Ellermeier and Slauch 2006; Grimont and Weill 2007). Since each serovar of *S. enterica* possesses unique genotypic and phenotypic characteristics, serovar identification is considered an initial step for investigating *S. enterica* related cases (Salazar et al. 2015).

The standardized White-Kauffman-Le-Minor scheme has been applied for phenotypic serovar identification based on the agglutination reaction of fresh bacterial culture to various O- and H- antisera. For genotyping, pulsed-field gel electrophoresis (PFGE) has been applied in diagnostic laboratories since 1993 in the United States and since 2000 in Canada by PulseNet, which is the electronic network of DNA fingerprinting database (<https://www.nml-lnm.gc.ca/Pulsenet/index-eng.htm>). PFGE is considered the gold standard molecular method for tracking the source of *Salmonella* infections (Wattiau et al. 2011). In recent years, whole-genome sequencing (WGS) has begun to replace PFGE as a molecular diagnostics method, to better understand the organisms by analysing genome rearrangement such as indels and single nucleotide polymorphism or variation (SNP/SNV) and comparison to reference genomes (Carleton and Gerner-Smidt 2016; Ronholm et al. 2016). However, since these methods are expensive, time-consuming, and laborious, many hospitals and private laboratories need to ship samples to reference laboratories due to lack of expertise and this causes serious delays in case of foodborne outbreak investigation.

Pyrosequencing is one of the most rapid DNA sequencing techniques with the ability to obtain relatively short read sequences of 40-80 bases in an hour following PCR amplification (Ronaghi 2001). Pyrosequencing of PCR products provides a means of confirming the identity of amplicons to ensure

reliability of test results for biothreat agents such as *Yersinia pestis* (Amoako et al. 2012) and *Bacillus anthracis* (Janzen et al. 2015). Furthermore, by carefully selecting sequencing locations containing characteristic SNPs, pyrosequencing assay enables analysis of sequence variations linked to phylogenetic identity of isolates, as demonstrated previously with Shiga-toxigenic *E. coli* (STEC) isolates including O157:H7 and other priority STEC serotypes (Goji et al. 2015). In this study, we developed a novel assay for *S. enterica* subsp. *enterica* serovar identification, aiming at differentiating the most common serovars in Canada. A triplex PCR and subsequent pyrosequencing was designed based on the detection of SNPs on one gene coding a surface protein (*fliD*) and two virulence genes (*sopE2* and *spaO*). The entire procedure, from an unknown bacterial colony on a culture plate to serovar identification can be completed in less than 5 hours at a fraction of the cost of whole genome sequencing.

Materials and Methods

Target serovars for pyrosequencing assays and bacterial strains

The pyrosequencing assay was designed to differentiate the top 10 common *S. enterica* serovars reported to the National Enteric Surveillance Program (NESP) in Canada in 2014 (<http://publications.gc.ca/site/eng/432850/publication.html>), namely *S. enterica* serovar Enteritidis, Typhimurium, ssp I 4,[5],12:i:-, Heidelberg, Thompson, Newport, Infantis, Typhi, Javiana, and Saintpaul. These top 10 serovars represent approximately 71-77% of all *Salmonella* isolates reported to NESP from 2009 to 2014. Additional 44 common serovars of *S. enterica* subsp. *enterica* plus *S. enterica* subsp. *salamae* and *S. enterica* subsp. *arizonae* were also tested (Table 1). The total of 54 *S. enterica* serovars selected comprises approximately 90-93% of all *Salmonella* isolates reported to NESP from 2009 to 2014 (data not shown).

All bacterial strains tested in this study are listed in Table 1. A total of 128 *S. enterica* isolates from foods, animal feces, and environment were obtained from Calgary, Ottawa-Fallowfield and Lethbridge Laboratories of the Canadian Food Inspection Agency (CFIA). *S. bongori* and 7 non-*Salmonella* bacteria were also tested (Table 1). Thirteen serovars were only tested *in silico* by the genome

sequences obtained from the National Centre for Biotechnology Information (NCBI) database including ssp I 4,[5],12:i:-, Javiana, Berta, Stanley, Uganda, Hartford, Paratyphi B, Bareilly, Manhattan, Paratyphi A, Litchfield, Virchow and Eastbourne (GenBank accession numbers are available in supplementary Table S1). Serovar identification was conducted either through traditional serological testing by the National Microbiology Laboratory at Guelph, Public Health Agency of Canada, or PFGE by the CFIA Ottawa Fallowfield laboratory. To verify the serovars, the *de novo* whole genome sequence assembly data of each isolate (unpublished) was applied to *Salmonella in silico* Typing Resource (SISTR) – an online bioinformatics platform for rapidly performing simultaneous *in silico* analyses (Yoshida et al. 2016).

PCR assay design and triplex PCR conditions

The NCBI Basic Local Alignment Search Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and Geneious sequence search tool on Geneious R9 software (Biomatters, Auckland, New Zealand) were applied to assemble and align reference sequences for known virulence genes and genes coding surface proteins to identify target suitable SNPs. As a result three genes – *fliD*, *sopE2* and *spaO* were selected. Primers to amplify each targeted region were designed using the PyroMark Assay Design software version 2.0.1 (Qiagen Inc., Hilden, Germany) based on the sequence analysis method (SQA) and the primers selected for this study are listed in Table 2. The specificity of the primers was verified using the NCBI primer-BLAST.

As shown in Figure 1, *fliD* genes of the serovars tested were classified into three different sequence types, namely *fliD*-ST (representing serovars Typhimurium type), *fliD*-SE (Enteritidis) and *fliD*-SC (Cerro). A triplex PCR reaction was set up to amplify, *fliD*-ST, *sopE2*, and *spaO*. All samples negative for *fliD*-ST were then confirmed by singleplex PCR, using a specific primer pair designed to amplify the *fliD*-SE and *fliD*-SC types.

The triplex PCR was carried out in a 50 µL PCR reaction mix containing 2x master mix of LightCycler® 480 Probes Master (Roche Diagnostics, Basel, Switzerland), primers with final concentrations specified in Table 2, and 2 µL of DNA template extracted by the colony boiling method as previously described (Goji et al. 2015). The *fliD*-SE/SC singleplex PCR was performed in a 12.5 µL PCR

reaction using 1 μ L of DNA template. PCR reactions were performed as follows: denaturation at 95 °C for 5 min, followed by 45 cycles of 95, 58, and 72 °C for 30 s each, and a final extension at 72 °C for 10 min. PCR amplicons were visualized using a QIAxcel DNA Screening Kit (Qiagen Inc.) on the QIAxcel capillary gel electrophoresis system (Qiagen Inc.). Samples showing the expected amplicon sizes were tested with downstream pyrosequencing assays.

Pyrosequencing and Data analysis

Two sequencing primers for each target gene were designed to amplify characteristic SNPs using the PyroMark Assay Design software (Table 2). Pyrosequencing was conducted using the PyroMark Q24 system (Qiagen Inc.) following the previously reported method (Goji et al. 2015). The dispensation orders designed for each sequencing primer are listed on Table S2. The raw pyrogram data from the instrument was transferred to the PyroMark Q24 software (version 2.0.6; Qiagen Inc.) and subsequently to Geneious software to align the sequence reads to the references for SNP/SNV calling. GenBank accession numbers of the reference genomes used in this study are listed in Table S1. Subgroup nomenclature represent the SNP/SNV variations of each gene in either numeric or alphabetical orders (i.e. serovar Thompson is Subgroup **T1Aa**, **T1** represents the SNP/SNV combination type-1 of *fliD*-ST, capitalized alphabet **A** is for combination type-A of *sopE2* and the last small letter **a** is for combination type-a of *spaO*. The names of the subgroups *fliD*-SE/SC have no combination numbers for *fliD* since it was not analyzed in this study (i.e. serovar Enteritidis is Subgroup **ECe**). The 23 SNPs tested in this study were concatenated to generate a phylogenetic tree using the Geneious Tree Builder of the Geneious software with the application of the Tamura-Nei model on Neighbor-Joining method.

Results and Discussion

PCR amplification

Virulence gene sequences of *S. enterica* were assembled and used for the selection of pyrosequencing targets. Multiple alignments of these genes were performed for 10 serovars and primers flanking targets containing the canonical SNPs and SNVs were designed. Based on these alignments,

149 three genes—*fliD*, *sopE2* and *spaO* – were selected and 2 sets of sequencing primers for each gene were
150 designed to analyze a total of 23 SNPs/SNVs (Table 3).

151 The *fliD* gene, also known as *flaV*, encodes a flagellar filament capping protein (Ikeda et al.
152 1987). Other flagellar genes, *fliC* and *fliB* that correlate with H antigen expression, have been applied to
153 previous studies for molecular serotyping of *S. enterica* (Leader et al. 2009; Mortimer et al. 2004; Ranieri
154 et al. 2013). We found three distinct sequence variations in *fliD* genes among *S. enterica* and classified
155 them into three types, *fliD*-ST (representing Typhimurium), *fliD*-SE (Enteritidis) and *fliD*-SC (Cerro)
156 (Figure 1). As the serovar Enteritidis and Typhimurium are the first and third most common serovars in
157 Canada and all the remaining top 10 common serovars belong to *fliD*-ST group, the multiplex PCR was
158 designed to specifically amplify *fliD*-ST type. A singleplex *fliD*-SE/SC PCR was also designed to confirm
159 the presence of *fliD* gene of both *fliD*-SE and *fliD*-SC types. *sopE2* and *spaO* genes encode proteins
160 involved in the type III secretion system (T3SS). SopE2 protein is secreted by T3SS that promotes actin
161 rearrangement of host cell to assist *Salmonella* invasion (Stender et al. 2000). Lara-Tejero et al.
162 demonstrated that the SpaO protein is a component of sorting platform, which is required to translocate
163 proteins to host cell through the T3SS in correct order (Lara-Tejero et al. 2011).

164 All *S. enterica* isolates and the two *S. enterica* subspecies (*S. enterica* subsp. *salamae* and
165 *arizonae*) tested in this study generated either two (*spaO* and *sopE2*) or all three (*spaO*, *sopE2*, and *fliD*-
166 ST) amplicons of expected sizes with the triplex PCR (Table 1). Forty one *S. enterica* serovars and *S.*
167 *enterica* subsp. *salamae* were positive with *fliD*-ST PCR and all 13 *fliD*-ST-negative serovars were
168 positive by confirmatory *fliD*-SE/SC PCR (Table 1). The *fliD*-SE/SC type serovars included Enteritidis,
169 Dublin, Berta, Rissen, Derby, Senftenberg, Havana, Albany, Agona, Cubana, Montevideo, Tennessee,
170 and Oranienburg. *S. enterica* subsp. *arizonae* was also *fliD*-SE/SC type (Table 1). Three other *S. enterica*
171 subspecies, namely *indica*, *houtenae* and *diarizonae* were also tested *in silico*. *S. enterica* subsp. *indica*
172 and *diarizonae* were *fliD*-ST positive while *S. enterica* subsp. *houtenae* was *fliD*-SE/SC positive (Table
173 1). It may be worth noting that the *fliD* genes of *S. arizonae*, *houtenae* and *salamae* are truncated either

due to a frame shift or a non-synonymous SNP and may not fully function as a protein (data not shown). *S. bongori* and the 7 non-*Salmonella* bacteria were all negative with the triplex PCR (Table 1).

SNP analysis of top 10 common *S. enterica* serovars in Canada

The results of the 23 SNPs/SNVs in the three genes tested by either pyrosequencing or *in silico* are shown in Table 3. Ten SNPs/SNVs are located on *fliD*-ST, seven on *sopE2*, and six on *spaO*. The SNP variations clustered all 54 serovars into 54 subgroups (SG). The non-synonymous C/A change at the 73rd nucleotide of *spaO* gene was unique among *S. enterica* subsp. *enterica* and could be used to distinguish *S. enterica* from other *Salmonella* subspecies (Table 3). It may be worth noting that the quality of the *fliD* pyrosequencing result of *S. salamae* was inadequate for base calling, likely due to a single mismatch on the locations of both *fliD* sequencing primers (position 602 and 729), thus the PCR positive and unsuccessful pyrosequencing result indicate the isolate is not *S. enterica*.

Among the Canadian top 10 common serovars, Newport, Typhi, Javiana, Infantis, Thompson, Heidelberg and Enteritidis demonstrated unique SNP/SNV combinations and were distinguished from other serovars tested in this study. The serovar Enteritidis is the only serovar that was negative to *fliD*-ST PCR among the 10 common serovars (Table 1). Also, the C/A SNP at the 455th nucleotide of *sopE2* (Table 3) discriminated Enteritidis (SG-ECe) from the other 53 serovars examined in this study. Allard et al. previously reported this SNP as unique to Enteritidis (Allard et al. 2013).

All Saintpaul isolates tested in this study clustered together except Saintpaul str. SARA29. Within our 23 SNPs/SNVs tested, this isolate was found to have a distinct SNP variation and generated a unique cluster, SG-T7Hd (Table 3). This result supports previous phylogenetic analyses reported by Timme et al, using whole genome kSNP analysis with a new reference-free k-mer approach of gathering SNPs from whole genome sequencing of 156 isolates representing 78 serovars (Timme et al. 2013), as well as the Multilocus Sequence Typing (MLST) application for serovar identification by Achtman et al. (Achtman et al. 2013)

Serovar Newport is polyphyletic and grouped into 3 lineages; lineage I, II, and III using MLST data of 384 Newport isolates from across the globe (Sangal et al. 2010). Lineage II is further subdivided

200 into II-A, -B, and -C by Cao et al, who performed phylogenetic analyses using over 147,000 SNPs from
201 26 diverse Newport isolates (Cao et al. 2013). In this study, two Canadian isolates of serovar Newport
202 were examined with pyrosequencing and 22 reference strains were analysed *in silico*. The pyrosequencing
203 assay successfully differentiated serovar Newport from others and further divided the 24 isolates into 5
204 subgroups; SG-T4Ai, -T4Ba, -T2Ba, -T7Hi and -T7Hc (Table 3). SG-T4Ai strains belong to lineage II-
205 A/C and SG-T4Ba to lineage II-B. SG-T2Ba is identical to lineage III. Isolates belonging to Lineage I
206 (SG-T7Hi and -T7Hc) have been reported mainly in European countries but not in North America (Sangal
207 et al. 2010), in fact the two Canadian isolates tested in this study were lineage II-A/C and III. Achtman et
208 al. examined concordance between MLST and traditional serotyping (Achtman et al. 2012), differentiated
209 serovar Newport into 4 e-Burst Groups (eBG) – clonal complexes of related sequence types. There was a
210 high degree of agreement between the four eBG and the five subgroups generated in our study. The eBG3
211 represented SG-T4Ai, eBG7 was SG-T4Ba, and eBG2 was SG-T2Ba. Some of the lineage I isolates
212 including str. 45982 that belong to eBG35 represented SG-T7Hc, by the A/G synonymous SNP at 753rd
213 nucleotide of *fliD* gene, while the majority of the lineage I isolates (eBG35) were ST-T7Hi.

214 *S. enterica* serovar Typhi, the causative agent of Typhoid fever, causes potentially fatal infection
215 only in human species and is known to be a relatively new monophyletic serovar (Dougan and Baker
216 2014). The targeted SNP/SNV assay presented in this study demonstrates that Typhi was not only
217 discriminated from all other serovars tested in this study, but also splits Typhi into two subgroups, SG-
218 T5Bc and -T5Dc by a non-synonymous SNP at the 463rd nucleotide of *sopE2* (A/G). In a previous study
219 by Holt et al. that analyzed 1,964 SNPs of 19 *S. enterica* serovar Typhi isolates representing 13
220 haplotypes, this SNP was presented as one of the conserved SNPs among haplotypes including H10 and
221 H58 that branched out after ST20b phage insertion event (Holt et al. 2008). H58 is an emerging haplotype
222 in Asia and Africa that is known to be resistant to nalidixic acid and have reduced susceptibility to
223 fluoroquinolones (Dougan and Baker 2014). It must be noted that the *sopE2* gene of serovar Typhi is a
224 pseudogene due to the loss of one adenosine near nucleotides 68 to 73 (data not shown) and it has not
225 been selected as a canonical SNP in the recent report analyzing WGS of over 2,000 Typhi isolates (Wong

et al. 2016). Nevertheless, this unique SNP at the 463rd nucleotide of *sopE2* would be applicable to differentiate the emerging subgroup classified into SG-T5Dc.

All four Canadian isolates of serovar Infantis that were pyrosequenced and six other isolates analysed *in silico* were classified into one group (SG-T3Ia) except str. SARB27 (Table 3). SARB27 has been recognized as a rare mutation of Infantis isolated from Senegal (Porwollik et al. 2004) and derived from a unique subgroup in this study (SG-T1Gf). To further characterize serovar Infantis, Yokoyama et al. performed phylogenetic analysis and divided the serovar into five clusters (Yokoyama et al. 2015).

Typhimurium and ssp I 4,[5],12:i:- yielded the same SNP pattern and were clustered into the same subgroup, SG-T6Ac (Table 3). Several genotypic analyses previously performed have found the genetic relatedness of the two serovars (den Bakker et al. 2011; Leekitcharoenphon et al. 2012) and ssp I 4,[5],12:i:- has been recognized as a monophasic variant of Typhimurium (Hayden et al. 2016; Lucarelli et al. 2010). These two serovars have 100% identity in the three genes we targeted in this study. Ido et al. tested 51 isolates of ssp I 4,[5],12:i:- from Japan using comparative genomic hybridization analysis and reported that the deletion and point mutations on the operon of phase 2 flagellin expression (*fljA*, *fljB*, and *hin*) were the major genetic modification of the monophasic phenotype of ssp I 4,[5],12:i:- from serovar Typhimurium (Ido et al. 2014).

Other common serovars in Canada and their phylogenetic analysis

A total of 41 *fliD*-ST type serovars were tested in this study and classified into 12 clusters (T1 - T12) by *fliD*-ST pyrosequencing. The *sopE2* and *spaO* pyrosequencing results further divided them into 42 subgroups (Table 3). Each of thirty-three subgroups corresponded to only one serovar, while the other 9 subgroups contained 2 or more representative serovars. The 13 *fliD*-SE-type serovars including Enteritidis listed on Table 3 were classified into 6 clusters by *sopE2* and further divided into 12 subgroups by *spaO* SNPs/SNVs. The *spaO*-68th SNP (T) was unique to serovar Agona among the serovars tested in this study. The names of serovars that were divided into more than one subgroup as well as subgroups representing more than one serovar are highlighted on Table 3.

In order to examine the phylogenetic relatedness of the subgroups generated in this study, a phylogenetic tree was constructed with the concatenated 23 SNP sequences (Figure 2) and compared to the tree that was generated by non-referencebased WGS SNP assay comprising >100,000 SNPs with 95% majority matrix among 78 serovars (Timme et al. 2013). The serovars were classified into the three major *S. enterica* clades (A1, A2, B) as reported by Timme et al. except serovars Tennessee, Weltevreden, and Oranienburg (Fig. 2). Some isolates from the polyphyletic serovars were clustered into different clades including Infantis str. SARB27 and Muenchen str. baa1674. These ambiguities observed from the comparison of this study to the WGS analyses may be easily explained by the less discriminating power of the pyrosequencing assay platform, due to the limited number of SNPs examined especially with *fliD*-SE type serovars, showing only 13 nucleotides from two genes.

In the WGS SNP analyses by Timme et al, the “section Typhimurium” consists of serovar Typhimurium, ssp I 4,[5],12:i-:, Saintpaul, Heidelberg and Paratyphi B str. SARA42. The serovars Heidelberg and Paratyphi B str. SARA42 share the same O and H2 antigen types and were clustered closely together but separated from the other serovars of “section Typhimurium” in our assay. Serovars Stanley and Anatum shared only some characteristics of O:H antigens (Stanley O:1,4,[5],12,[27] and Anatum H1:e.h.) and clustered into SG-T6Hc along with Saintpaul. In case further investigation is required to identify “section Typhimurium”, it would not be so challenging to locate unique SNPs/SNVs and design additional pyrosequencing assays to differentiate Stanley and Anatum from Saintpaul, since they diverged from different branches in the Clade A2. The divergence of the phylogenetic tree constructed in this study provides an in depth insight of the phylogeny of serovars including Newport, Kentucky, Give, Paratyphi B, Muenchen, Montevideo, Senftenburg, and Bareilly. One Canadian isolate and fourteen genomes of serovar Montevideo were examined *in silico* and clustered into three separate branches (SG-EEc, -EEe and -EEf). Allard et al. divided 47 Montevideo isolates into four lineages (Clade I to IV) based on an analysis of 72,063 variable SNP sites. Isolates from SG-EEc in this study were categorized into Clades I and IV, whereas SG-EEe and -EEf were Clades II and III respectively (Table 3). One Canadian isolate pyrosequenced was in the same group of str. LQC10, SG-EEe (Clade II). From

another perspective, it might be noteworthy that a recent study showed that *spaO* and *sopE2* were in the group of genes induced after enrichment in the mucoid variant compared to the non-mucoid variant of serovar Montevideo (Jean-Gilles Beaubrun et al. 2017). Since all SNVs in the *spaO* gene examined in this study are all non-synonymous (Table 3), these may provoke significant changes in biochemical and virulence characteristics. Serovar Muenchen was divaricated into three subgroups (SG-T7Gc, -T7Fc and -T7Fi) and two Canadian isolates tested with pyrosequencing were SG-T7Gc, which includes other serovars, namely Paratyphi A, Mbandaka and Indiana in Clade A2 (Table 3 and Fig. 2).

Pyrosequencing application for foodborne pathogen investigation

In order to respond to foodborne illness cases in a timely manner, rapid, cost-effective, and confirmatory detection and identification methods are required. The pyrosequencing assay developed in this study can be completed in 5 hours from an unknown colony on a culture plate to the sequence results and analyses, thus the turn-around time is significantly shorter than most of the sequencing platforms currently available. Besides, the labour and reagent costs are relatively inexpensive (about \$30 Canadian for testing all 6 targets). The boiling method used for DNA extraction is simple and quick, and applicable to other Enterobacteriaceae. The single-tube, multiplex PCR developed for three target genes requires minimal sample preparation labour. Pyrosequencing results can be obtained in real-time and the relatively short sequence reads up to 50 bp does not demand a high-speed computer with expensive data analysis applications or an experienced bioinformatician. In addition, the pyrosequence data can be confirmatory and epidemiologically valuable.

There have been several previous reports on the application of pyrosequencing to *Salmonella* detection. Mortimer et al. selected *fliC* alleles to identify approximately 97% of the 50 medically most important *Salmonella* in England and Wales (Mortimer et al. 2004). However, since they sequenced the entire *fliC* gene, the protocol was laborious using many sequencing primers and did not distinguish important serovars such as Enteritidis. Li et al. applied pyrosequencing for *Salmonella* rapid identification, although this method was not meant to differentiate serovars (Li et al. 2012).

Real-time PCR has also been applied as a quick identification tool for *Salmonella* serovars (Maurischat et al. 2015; Park and Rieke 2015; Prendergast et al. 2013), though this application has limitations with the number of serovars detected simultaneously, due to the usage of fluorescent DNA-intercalating dye for probes. A 16-plex PCR was also developed previously to discriminate the most common serovars of *S. enterica* in the United States (Leader et al. 2009). In the report, 751 serotyped isolates representing 50 serovars were tested in a blinded study and 32 of the serovars matched the classical serotyping results. PCR is rapid and easily accessible to most of the modern testing laboratories, however not only 64 % of accuracy of the multiplex PCR results is unreliable but also due to the ambiguity of PCR reaction, the results have been treated as presumptive and require further investigations. Meanwhile, pyrosequencing generates sequence results which provide an added layer of confidence to PCR results.

Despite the development of a global transportation network of food and animals, some *S. enterica* serovars exhibit geographic trends in their distribution. As we demonstrate here, to differentiate common *S. enterica* serovars to Canada, this assay platform is easily and quickly applied to target common serovars of any other areas or to detect emerging isolates in foodborne disease outbreak scenarios, by setting up new assays with targeted SNPs/SNVs. The availability of publicly accessible whole genome sequence data and the current advancement in the computational sequence data analysis have made it possible to select target genes and develop a new pyrosequencing assay. The mounting evidence in the strong discriminating power of WGS is moving this technology towards becoming the gold standard in developed countries including Canada for foodborne pathogen investigation (Ronholm et al. 2016). However, an initial quick screening/discriminative test is needed for situations involving large submissions from the same foodborne disease outbreak, which requires a short processing time. Pyrosequencing will also provide a useful application for routine environmental monitoring with a reduced cost.

Conclusion

The combined multiplex PCR-pyrosequencing assay developed in this study differentiated seven of the most common serovars of *S. enterica* including Thompson, Enteritidis, Newport, Typhi, Javiana, Heidelberg, and Infantis among a total of 54 common serovars in Canada. Furthermore the 54 serovars were divided into 54 subgroups that indicate some potential epidemiological propensities of the pathogen. Due to the fact that *S. enterica* is ubiquitous in the environment and many serovars have a wide range of hosts, routine surveillance to monitor serovar distribution is critical for preventative action against foodborne disease caused by *Salmonella* spp. This pyrosequencing assay of *S. enterica* has great potential to be applied as a quick identification and confirmatory method for frontline laboratories during foodborne disease outbreak investigations, as well as an environmental screening tool for the food and animal industries.

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Table 1. Bacterial strains used in this study and triplex PCR results.

* *fliD*-ST PCR positive

† *fliD*-SE PCR positive

Table 2. Primers used in this study.

* Accession number: AE006468

† Location on *S. Enteritidis* str. P125109 (accession number AM933172)

‡ Not applicable.

Table 3. Twenty-three SNPs analysed by pyrosequencing assay. The names of serovars divided into more than one subgroup and subgroups representing more than one serovar were highlighted.

*C; cytosine, T; thymine, A; adenine, G; guanine

†; Nucleotide location on the coding gene. Bold numbers indicate non-synonymous SNPs

Figure 1. Alignment of *fliD* genes.

Each GenBank accession number is shown on the top of each serovar name. Highlighted nucleotides indicate sequence variations. Nucleotide numbering is based on the *fliD* coding sequence of *S. Typhimurium* str. LT2. Two pairs of primers and two sequencing primers used in this study are also presented.

Figure 2. Phylogenetic tree constructed from the identified 23 SNPs based on the Neighbor-Joining method. The classification of *Salmonella enterica* Clade A1, A2 and B by Timme et al. was applied to the serovars tested in this study and are color-coded.

Table S1. Reference strains and accession numbers.

Table S2. Dispensation orders for pyrosequencing used in this study.

Species	number of isolates		<i>fliD</i>	<i>sopE2</i>	<i>spaO</i>
	PCR	<i>in silico</i>			
10 common serovars of <i>S. enterica</i>					
<i>S. Enteritidis</i>	32	5	SE/SC*	+	+
<i>S. Heidelberg</i>	16	6	ST†	+	+
<i>S. Typhimurium</i>	24	5	ST	+	+
<i>S. ssp I 4,[5],12:i:-</i>	0	4	ST	+	+
<i>S. Newport</i>	2	22	ST	+	+
<i>S. Thompson</i>	4	4	ST	+	+
<i>S. Typhi</i>	1	4	ST	+	+
<i>S. Infantis</i>	4	5	ST	+	+
<i>S. Javiana</i>	0	5	ST	+	+
<i>S. Saintpaul</i>	2	4	ST	+	+
additional 44 common serovars					
<i>S. Dublin</i>	1	3	SE/SC	+	+
<i>S. Berta</i>	0	1	SE/SC	+	+
<i>S. Rissen</i>	1	1	SE/SC	+	+
<i>S. Senftenberg</i>	1	6	SE/SC	+	+
<i>S. Derby</i>	2	1	SE/SC	+	+
<i>S. Havana</i>	1	1	SE/SC	+	+
<i>S. Albany</i>	2	1	SE/SC	+	+
<i>S. Agona</i>	2	3	SE/SC	+	+
<i>S. Cubana</i>	1	2	SE/SC	+	+
<i>S. Montevideo</i>	1	14	SE/SC	+	+
<i>S. Tennessee</i>	1	3	SE/SC	+	+
<i>S. Oranienburg</i>	1	3	SE/SC	+	+
<i>S. Hadar</i>	1	2	ST	+	+
<i>S. Anatum</i>	1	3	ST	+	+
<i>S. Stanley</i>	0	1	ST	+	+
<i>S. Uganda</i>	0	1	ST	+	+
<i>S. Brunei</i>	1	1	ST	+	+
<i>S. Hartford</i>	0	2	ST	+	+
<i>S. Panama</i>	1	1	ST	+	+
<i>S. Give</i>	2	3	ST	+	+
<i>S. Weltevreden</i>	1	4	ST	+	+
<i>S. Paratyphi B</i>	0	9	ST	+	+
<i>S. Chester</i>	1	1	ST	+	+
<i>S. Bareilly</i>	0	5	ST	+	+
<i>S. Mbandaka</i>	2	3	ST	+	+
<i>S. Kentucky</i>	3	4	ST	+	+
<i>S. Manhattan</i>	0	2	ST	+	+
<i>S. London</i>	1	1	ST	+	+
<i>S. Paratyphi A</i>	0	3	ST	+	+
<i>S. Muenchen</i>	2	6	ST	+	+
<i>S. Indiana</i>	1	1	ST	+	+
<i>S. Braenderup</i>	3	2	ST	+	+
<i>S. Bovismorbificans</i>	2	1	ST	+	+

<i>S. Litchfield</i>	0	2	ST	+	+
<i>S. Choleraesuis</i>	1	4	ST	+	+
<i>S. Paratyphi C</i>	1	1	ST	+	+
<i>S. Virchow</i>	0	2	ST	+	+
<i>S. Schwarzengrund</i>	1	2	ST	+	+
<i>S. Brandenburg</i>	1	4	ST	+	+
<i>S. Sandiego</i>	1	2	ST	+	+
<i>S. Eastbourne</i>	0	1	ST	+	+
<i>S. Poona</i>	1	1	ST	+	+
<i>S. Muenster</i>	1	2	ST	+	+
Other <i>S. enterica</i> subspecies					
<i>S. enterica</i> subsp. <i>salamae</i>	1	1	ST	+	+
<i>S. enterica</i> subsp. <i>indica</i>	0	1	ST	±	+
<i>S. enterica</i> subsp. <i>diarizonae</i>	0	1	ST	+	+
<i>S. enterica</i> subsp. <i>arizonae</i>	1	1	SE/SC	+	+
<i>S. enterica</i> subsp. <i>houtenae</i>	0	1	SE/SC	±	+
Non-<i>S. enterica</i> bacteria					
<i>Salmonella bongori</i>	1	1	-	-	-
<i>Hafnia alvei</i>	1	-	-	-	-
<i>Shigella sonnei</i>	1	-	-	-	-
<i>Shigella dysenteriae</i>	1	-	-	-	-
<i>Klebsiella pneumoniae</i>	1	-	-	-	-
<i>Pseudomonas aeruginosa</i>	1	-	-	-	-
<i>Escherichia coli</i>	2	-	-	-	-
Total number of isolates	138	186			

* *fliD* -ST PCR positive

† *fliD* -SE PCR positive

Primer names	Sequences	Amplicon size (bp)
spaO_Fwd_Bio	/5Biotin/ GCA AAC CGC GAC AGA ATG C	155
spaO_Rvs	CCA GAG CGG GAGAGACATGC	
sopE2_Fwd_Bio	/5Biotin/ TCA GAC ATG CGA AGC CAT RTT A	290
sopE2_Rvs	CGA TTT TCT CAG CAT RCG CTT TA	
fliD-ST_Fwd	CGA CAA TAC GAT GAA GAT CAC G	250
fliD-ST_Rvs_Bio	/5Biotin/ CGC CTT GGT ATC ATC TTT CGT	
fliD-SE/SC_Fwd	AAG CGC TGG ATG AAA ATA CCG C	120
fliD-SE/SC_Rvs	TAA TRC CGT CAT CSG CCA GAT	
spaO_seq1	TCA AGC CAG TCC CCA G	N/A‡
spaO_seq2	TCG GAT ATT CCA GCG	N/A
sopE2_seq1	CTG CCC CAC CTG GAG	N/A
sopE2_seq2	GCC TCT CCT ATC ACT TTC A	N/A
fliD-ST_seq1	AAG GCG ATA CAA AAC T	N/A
fliD-ST_seq2	TAA CCG ACG CCC CTC	N/A

* accession number AE006468.2
† Location on S. Enteritidis str. P125109 (accession number AM933172)
‡ Not applicable.

Concentration in reaction (μM)	Annealing temperature ($^{\circ}\text{C}$)	Location on <i>S. Typhimurium</i> str. LT2*
0.3	58	3035206-3035188
0.3	58	3035052-3035071
0.4	58	1952408-1952387
0.4	58	1952119-1952141
0.2	58	2049965-2049986
0.2	58	2050214-2050194
0.6	58	1145251-114523†
0.6	58	1145132-1145152†
N/A	N/A	3035072-3035087
N/A	N/A	3035151-3035165
N/A	N/A	1952230-1952244
N/A	N/A	1952292-1952310
N/A	N/A	2049991-2050006
N/A	N/A	2050126-2050140

gene name		<i>fliD</i> -ST							
<i>S. enterica</i> serovars	Subgroup	612†	616	639	651	747	748	753	758
Thompson	T1Aa	C*	T*	C	T	A*	A	G*	C
Infantis (str. SARB27)	T1Gf	C	T	C	T	A	A	G	C
Newport (Lineage III, eBG2)	T2Ba	C	T	C	T	A	A	A	C
Heidelberg	T3Aa	C	C	T	T	A	A	A	C
Infantis	T3Ia	C	C	T	T	A	A	A	C
Newport (Lineage II-A and C, eBG3)	T4Ai	C	C	C	T	A	A	A	C
Newport (Lineage II-B, eBG7)	T4Ba	C	C	C	T	A	A	A	C
Typhi (str. CT18)	T5Bc	C	C	C	T	A	A	A	C
Typhi (str. Ty2)	T5Dc	C	C	C	T	A	A	A	C
Javiana	T5Ee	C	C	C	T	A	A	A	C
Typhimurium	T6Ac	C	C	C	T	A	A	G	C
4,[5],12:i:-	T6Ac	C	C	C	T	A	A	G	C
Saintpaul	T6Hc	C	C	C	T	A	A	G	C
Saintpaul (str. SARA29)	T7Hd	C	C	C	T	A	A	G	C
Newport (Lineage I, str 45982, eBG35)	T7Hc	C	C	C	T	A	A	G	C
Newport (Lineage I, eBG35)	T7Hi	C	C	C	T	A	A	G	C
Enteritidis	ECe								
Kentucky	T1Gb	C	T	C	T	A	A	G	C
Paratyphi B (str. SARA62)	T1Gd	C	T	C	T	A	A	G	C
Mbandaka	T1Gh	C	T	C	T	A	A	G	C
Hadar	T1Ha	C	T	C	T	A	A	G	C
London	T1Hc	C	T	C	T	A	A	G	C
Manhattan	T1Hc	C	T	C	T	A	A	G	C
Weltevreden	T1Ia	C	T	C	T	A	A	G	C
Paratyphi B (str. SARA42)	T3Ha	C	C	T	T	A	A	A	C
Kentucky (str. ATCC9263)	T4Gh	C	C	C	T	A	A	A	C
Hartford	T4Ha	C	C	C	T	A	A	A	C
Brunei	T4Hc	C	C	C	T	A	A	A	C
Brandenburg	T5Ef	C	C	C	T	A	A	A	C
Give (str. S5-487)	T5Ef	C	C	C	T	A	A	A	C
Panama	T5Eg	C	C	C	T	A	A	A	C
Give (str.564)	T5Gf	C	C	C	T	A	A	A	C
Uganda	T6Ha	C	C	C	T	A	A	G	C
Anatum	T6Hc	C	C	C	T	A	A	G	C
Stanley	T6Hc	C	C	C	T	A	A	G	C
Bareilly (str. ATCC9115)	T6Ia	C	C	C	T	A	A	G	C
Paratyphi B var. Java	T6Ia	C	C	C	T	A	A	G	C
Muenchen (str. ATCC8388)	T7Fc	C	C	C	T	A	A	G	C
Muenchen (str. baa1674)	T7Fi	C	C	C	T	A	A	G	C
Paratyphi A	T7Gc	C	C	C	T	A	A	G	C
Muenchen (str. baa1594)	T7Gc	C	C	C	T	A	A	G	C
Mbandaka (str. ATCC51958)	T7Gc	C	C	C	T	A	A	G	C
Indiana	T7Gc	C	C	C	T	A	A	G	C
Braenderup	T7Hc	C	C	C	T	A	A	G	C
Bovismorbificans	T7Hc	C	C	C	T	A	A	G	C

Litchfield	T7Hc	C	C	C	T	A	A	G	C
Choleraesuis (str. ATCC10708)	T7Hc	C	C	C	T	A	A	G	C
Virchow	T7Ia	C	C	C	T	A	A	G	C
Choleraesuis	T7Ie	C	C	C	T	A	A	G	C
Paratyphi C	T7Ie	C	C	C	T	A	A	G	C
Bareilly (str. 2780)	T8Ic	C	C	C	T	A	A	A	C
Scwarzengrund	T9Ee	C	C	C	T	G	C	G	C
Paratyphi B (str. SPB7)	T10Ia	T	C	C	T	A	A	G	C
Paratyphi B (str. ATCC51962)	T11Ia	C	C	C	C	A	A	G	C
Muenster	T12Ee	C	C	C	C	A	A	A	C
Chester	T12Ee	C	C	C	C	A	A	A	C
Sandiego	T12Ef	C	C	C	C	A	A	A	C
Eastbourne	T12Ef	C	C	C	C	A	A	A	C
Poona	T12Eg	C	C	C	C	A	A	A	C
Dublin	EAe								
Tennessee	EEa								
Montevideo (str. LQC10)	EEc								
Montevideo (str. 4441H)	EEe								
Montevideo (str. ATCC BAA710)	EEf								
Cubana	EGc								
Rissen	EGd								
Derby	EGd								
Senftenberg (str. ATCC10708)	EGd								
Senftenberg (str. 604314)	EGh								
Havana	EGh								
Albany	EGh								
Agona	EGj								
Berta	EHe								
Oranienburg	EIe								
<i>S. enterica</i> subsp. <i>salamae</i>		C	T	C	C	A	A	G	A
<i>S. enterica</i> subsp. <i>diarizonae</i>		C	T	C	C	A	A	G	A
<i>S. enterica</i> subsp. <i>indica</i>	Non enterica	C	C	C	C	A	A	G	A
<i>S. enterica</i> subsp. <i>arizonae</i>									
<i>S. enterica</i> subsp. <i>houtenae</i>									

		<i>sopE2</i>								<i>spaO</i>					
765	786	421	455	463	465	471	477	480	68	73	80	87	156	163	
G	C	T	G	G	G	A	T	C	A	C	G	G	G	C	
G	C	C	G	G	G	A	C	C	A	C	A	T	G	A	
G	C	T	G	G	G	A	T	T	A	C	G	G	G	C	
G	C	T	G	G	G	A	T	C	A	C	G	G	G	C	
G	C	C	G	G	G	A	T	T	A	C	G	G	G	C	
G	C	T	G	G	G	A	T	C	A	C	A	G	G	C	
G	C	T	G	G	G	A	T	T	A	C	G	G	G	C	
C	C	T	G	G	G	A	T	T	A	C	G	G	C	A	
C	C	T	G	A	G	A	T	T	A	C	G	G	C	A	
C	C	C	G	G	A	G	C	C	A	C	A	T	G	C	
C	C	T	G	G	G	A	T	C	A	C	G	G	C	A	
C	C	T	G	G	G	A	T	C	A	C	G	G	C	A	
C	C	C	G	G	G	A	T	C	A	C	G	G	C	A	
G	C	C	G	G	G	A	T	C	A	C	G	T	C	A	
G	C	C	G	G	G	A	T	C	A	C	G	G	C	A	
G	C	C	G	G	G	A	T	C	A	C	A	G	G	C	
		T	A	G	G	A	T	C	A	C	A	T	G	C	
G	C	C	G	G	G	A	C	C	A	C	G	G	G	A	
G	C	C	G	G	G	A	C	C	A	C	G	T	C	A	
G	C	C	G	G	G	A	C	C	A	C	A	T	C	A	
G	C	C	G	G	G	A	T	C	A	C	G	G	G	C	
G	C	C	G	G	G	A	T	C	A	C	G	G	C	A	
G	C	C	G	G	G	A	T	C	A	C	G	G	C	A	
G	C	C	G	G	G	A	T	C	A	C	G	G	C	A	
G	C	C	G	G	G	A	T	C	A	C	G	G	C	A	
C	C	C	G	G	A	G	C	C	A	C	A	T	G	A	
C	C	C	G	G	A	G	C	C	A	C	A	T	G	A	
C	C	C	G	G	A	A	C	C	A	C	A	T	G	A	
C	C	C	G	G	G	A	T	C	A	C	G	G	G	C	
C	C	C	G	G	G	A	T	C	A	C	G	G	C	A	
C	C	C	G	G	G	A	T	C	A	C	G	G	C	A	
C	C	C	G	G	G	A	T	T	A	C	G	G	G	C	
C	C	C	G	G	G	A	T	T	A	C	G	G	G	C	
G	C	C	G	G	A	T	C	C	A	C	G	G	C	A	
G	C	C	G	G	A	T	C	C	A	C	A	G	G	C	
G	C	C	G	G	G	A	C	C	A	C	G	G	C	A	
G	C	C	G	G	G	A	C	C	A	C	G	G	C	A	
G	C	C	G	G	G	A	C	C	A	C	G	G	C	A	
G	C	C	G	G	G	A	C	C	A	C	G	G	C	A	
G	C	C	G	G	G	A	T	C	A	C	G	G	C	A	
G	C	C	G	G	G	A	T	C	A	C	G	G	C	A	

G	C	C	G	G	G	A	T	C	A	C	G	G	C	A
G	C	C	G	G	G	A	T	C	A	C	G	G	C	A
G	C	C	G	G	G	A	T	T	A	C	G	G	G	C
G	C	C	G	G	G	A	T	T	A	C	A	T	G	C
G	C	C	G	G	G	A	T	T	A	C	A	T	G	C
G	T	C	G	G	G	A	T	T	A	C	G	G	C	A
G	C	C	G	G	A	G	C	C	A	C	A	T	G	C
C	C	C	G	G	G	A	T	T	A	C	G	G	G	C
C	C	C	G	G	A	G	C	C	A	C	A	T	G	C
C	C	C	G	G	A	G	C	C	A	C	A	T	G	C
C	C	C	G	G	A	G	C	C	A	C	A	T	G	A
C	C	C	G	G	A	G	C	C	A	C	A	T	G	A
C	C	C	G	G	A	G	C	C	A	C	A	T	C	C
		T	G	G	G	A	T	C	A	C	A	T	G	C
		C	G	G	A	G	C	C	A	C	G	G	G	C
		C	G	G	A	G	C	C	A	C	G	G	C	A
		C	G	G	A	G	C	C	A	C	A	T	G	C
		C	G	G	A	G	C	C	A	C	A	T	G	A
		C	G	G	G	A	C	C	A	C	G	G	C	A
		C	G	G	G	A	C	C	A	C	G	T	C	A
		C	G	G	G	A	C	C	A	C	G	T	C	A
		C	G	G	G	A	C	C	A	C	A	T	C	A
		C	G	G	G	A	C	C	A	C	A	T	C	A
		C	G	G	G	A	C	C	A	C	A	T	C	A
		C	G	G	G	A	C	C	T	C	G	G	C	A
		C	G	G	G	A	T	C	A	C	A	T	G	C
		C	G	G	G	A	T	T	A	C	A	T	G	C
<hr/>														
G	C	C	G	G	A	A	C	C	A	A	A	T	C	A
G	C	C	G	G	A	A	T	C	A	A	A	T/A	C	A
G	C	C	G	G	A	A	C	C	A	A	A	T	C	A
		C	G	G	A	A	C	C	A	A	A	G	C	A
		C	G	G	A	A	C	C	A	A	A	T	C	A
<hr/>														

AE006468 (S.Typhimurium str. LT2)

AM933172 (S. Enteritidis str. P125109)
CP012833 (S. Cerro str. CFSAN001588)

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AE006468 (S.Typhimurium str. LT2)

AM933172 (S. Enteritidis str. P125109)
CP012833 (S. Cerro str. CFSAN001588)

Phylogenetic tree constructed from the identified 23 SNPs based on the Neighbor-Joining method. The classification of *Salmonella enterica* Clade A1, A2 and B by Timme et al. was applied to the serovars tested in this study and are color-coded.

493x423mm (72 x 72 DPI)

Canadian top 10 serovars of <i>S. enterica</i> subsp. <i>enterica</i>	Strain ID	Reference genome accession numbers	Reference
Enteritidis	P125109	AM933172	Thomson et al. 2008
	10-34587	AWOI000000000	den Bakker et al. 2014
	CDC 2010K-1882	ALFG000000000	Allard et al. 2013
	CDC 2010K-1884	ALFH000000000	Allard et al. 2013
	81-2625	ALIB000000000	Timme et al. 2013
Heidelberg	SL476	CP001120	Fricke et al. 2011
	SL486	ABEL000000000	Fricke et al. 2011
	str. 41578	CP004086	Hoffmann et al. 2014
	N18393	AMNF000000000	Hoffmann et al. 2014
	82-2052	AMMX000000000	Timme et al. 2013
Typhimurium	N15757	AMND000000000	Hoffmann et al. 2014
	LT2	AE006468	McClelland et al. 2001
	SL1344	FQ312003	Kroger et al. 2012
	14028S	CP001363	Jarvik et al. 2010
	UK-1	CP002614	Luo et al. 2011
I 4,[5],12:i:-	DT104	NC_022569	Mather et al. 2013
	CVM23701	NZ_ABAO000000000	Fricke et al. 2011
	08-1736	CP006602	Direct submission
	08-1700	AHUS000000000	Timme et al. 2013
	08-1739	AHUT000000000	Timme et al. 2013
Newport lineage I	45982	SRR1967865	Ashton et al. 2016
	32381	SRR3049464	Ashton et al. 2016
	56933	SRR1967090	Ashton et al. 2016
	89103	SRR1969927	Ashton et al. 2016
Newport lineage II-A	CVM19443	AHUB000000000	Cao et al. 2013
	CVM 19470	AHUE000000000	Cao et al. 2013
	CVM 19536	AHUF000000000	Cao et al. 2013
	CVM 4176	AHUG000000000	Cao et al. 2013
Newport lineage II-B	CVM19447	AHTP000000000	Cao et al. 2013
	CVM19449	AHTQ000000000	Cao et al. 2013
	CVM 19567	AHTR000000000	Cao et al. 2013
Newport lineage II-C	SL254	CP001113	Fricke et al. 2011
	CVM37978	AHUC000000000	Cao et al. 2013
	CVM 19593	AHUD000000000	Cao et al. 2013
Newport lineage III	SL317	ABEW000000000	Fricke et al. 2011
	CVM35199	AHTK000000000	Cao et al. 2013
	CVM21539	AHTL000000000	Cao et al. 2013
	CVM 33953	AHTM000000000	Cao et al. 2013
	CVM35188	AHTN000000000	Cao et al. 2013
	CVM 21559	AHTO000000000	Cao et al. 2013
	CVM 35202	AHTS000000000	Cao et al. 2013
	Levine 1	AJMN000000000	Cao et al. 2013
Thompson	RM1984	CP012513	Parker et al. 2015
	RM1986	CP012514	Parker et al. 2015

Typhi	RM6836	CP006717	Parker et al. 2013
	ATCC8391	CP011396	Timme et al. 2013
	CT18	AL513382	Parkhill et al. 2001
	Ty2	AE014613	Deng et al. 2003
	Ty21a	CP002099	Xu et al. 2013
Infantis	404ty	CAAQ01000000	Direct submission
	SARB27	NZ_CM001274	den Bakker et al. 2011
	str.120100	LMXO00000000	Direct submission
	FSL A4-543	JRXB00000000	Olasz et al. 2015
	SI 54/04	JRXC00000000	Olasz et al. 2015
Javiana	1326/28	NZ_LN649235	Olasz et al. 2015
	SI3337/12	MIJS00000000	Olasz et al. 2016
	CFSAN001992	CP004027	Allard et al. 2013
	GA_MM04042433	ABEH00000000.2	Fricke et al. 2011
	syt.10721	AOZA00000000	Timme et al. 2013
Saintpaul	ATCC BAA-1593	AHUW00000000	Timme et al. 2013
	PRS_2010_0720	AOZB00000000	Timme et al. 2013
	SARA23	ABAM00000000	Fricke et al. 2011
	SARA29	ABAN00000000	Fricke et al. 2011
	SARA26	AOXF00000000	Timme et al. 2013
	JO2008	AOXY00000000	Timme et al. 2013

44 additional common serovars

Dublin	SC50_1	LK931502	Direct submission
	SD3246	CM001151	Fricke et al. 2011
	CT_02021853	CP001144	Fricke et al. 2011
Berta	ATCC8392	NZ_AOZO00000000	Timme et al. 2013
Rissen	str. 150	FR775210	Timme et al. 2013
Senftenberg	FSL A4-543	AFCU01000000	den Bakker et al. 2011
	ATCC 43845	AOXX00000000	Timme et al. 2013
	ATCC 8400	AOXU00000000	Timme et al. 2013
	str. 316235162	AOYU00000000	Timme et al. 2013
	str. 423984-2	AOYP00000000	Timme et al. 2013
	str. 604314	AOXW00000000	Timme et al. 2013
Derby	str.626	AOZH00000000	Timme et al. 2013
Havana	CFSAN001082	APAH00000000	Timme et al. 2013
Albany	ATCC 51960	AOZW00000000	Timme et al. 2013
Agona	SL483	CP001138	Fricke et al. 2011
	648586-1	AOZU00000000	Timme et al. 2013
	ATCC 51957	AOZX00000000	Timme et al. 2013
	CFSAN002050	CP006055	Hoffmann et al. 2014
Cubana	CFSAN001083	APAG00000000	Timme et al. 2013
	str. 515920-1	AESL00000000	Allard et al. 2012
Montevideo	SARB31	AESR00000000	Allard et al. 2012
	ATCC BAA710	AESS00000000	Allard et al. 2012
	LQC 10	AEST00000000	Allard et al. 2012
	29N	AESW00000000	Allard et al. 2012

	42N	AESX00000000	Allard et al. 2012
	4441 H	AESY00000000	Allard et al. 2012
	str. 366867	AETC00000000	Allard et al. 2012
	str. 413180	AETD00000000	Allard et al. 2012
	str. 446600	AETE00000000	Allard et al. 2012
	str.556150-1	AETG00000000	Allard et al. 2012
	MB101509-0077	AETK00000000	Allard et al. 2012
	IA_2010008285	AETV00000000	Allard et al. 2012
	str. 8387	AOYQ00000000	Timme et al. 2013
Tennessee	CFSAN001378	CP014994	Direct submission
	TXSC_TXSC08-19	CP007505	Timme et al. 2013
	TXSC_TXSC08-21	AOXQ00000000	Timme et al. 2013
Oranienburg	str.701	AOYL00000000	Timme et al. 2013
	S-76	AR0Z00000000	Medrano-Felix et al. 2013
	str. 0250	AOYM00000000	Timme et al. 2013
Hadar	RI_05P066	ABFG00000000	Fricke et al. 2011
	ATCC 51956	AOZE00000000	Timme et al. 2013
Anatum	ATCC BAA-1592	CP007531	Timme et al. 2013
	USDA_100	APAA00000000	Timme et al. 2013
	USDA-ARS-USMARC-1766	CP014665	Fricke et al. 2011
Stanley	ATCC7308	NZ_AOXV00000000	Timme et al. 2013
Uganda	R8-3404	AFCV01000000	den Bakker et al. 2011
Brunei	20911	SRR3048885	Ashton et al. 2016
Hartford	06-0676	ALPO00000000	Direct submission
	CFSAN001075	APAO00000000	Timme et al. 2013
Panama	ATCC7378	CP012346	Timme et al. 2013
Give	FSL S5-487	AFCM01000000	den Bakker et al. 2011
	str.564	AOZG00000000	Timme et al. 2013
	CFSAN012622	JOKK00000000	Direct submission
Weltevreden	str.1655	CP014996	Direct submission
	2007-60-3289-1	FR775210	Brankatschk et al. 2011
	C2346	LN890520	Direct submission
	HI_N05-537	ABFF01000000	Fricke et al. 2011
Paratyphi B	SPB7	CP000886	Fricke et al. 2011
	ATCC51962	AOYC00000000	Timme et al. 2013
	ATCC19940	AOYD00000000	Timme et al. 2013
	ATCC10719	AOYF00000000	Timme et al. 2013
	ATCC 8759	AOYE00000000	Timme et al. 2013
	ATCC-BAA 1585	AOYG00000000	Timme et al. 2013
	SARA42	AOYB00000000	Timme et al. 2013
	SARA56	AOXH00000000	Timme et al. 2013
Chester	ATCC11997	NZ_AOZI00000000	Timme et al. 2013
Bareilly	CFSAN000189	CP006053	Timme et al. 2013
	ATCC9115	AOZN00000000	Timme et al. 2013
	CFSAN000197	AOZR00000000	Timme et al. 2013
	str. 2780	AOZP00000000	Timme et al. 2013

Mbandaka	CFSAN000200	AOZQ00000000	Timme et al. 2013
	ATCC 51958	AOYR00000000	Timme et al. 2013
	CVM N51315	LHOS00000000	Direct submission
	2012K-0273	ARYT00000000	Direct submission
Kentucky	CVM29188	ABAK02000001	Fricke et al. 2011
	CDC 191	ABEI00000000	Fricke et al. 2011
	ATCC 9263	AOYY00000000	Timme et al. 2013
	str. 5349	AOYZ00000000	Timme et al. 2013
Manhattan	CFSAN001078	NZ_APAL00000000	Timme et al. 2013
	str. 111113	NZ_CBKW020000018	Scaltriti et al. 2015
London	CVM N38882	JYXN00000000.1	Direct submission
Paratyphi A	ATCC9150	CP000026	Fricke et al. 2011
	AKU_12601	FM200053	Fricke et al. 2011
	ATCC11511	AOYH00000000	Timme et al. 2013
	ATCC 8388	AOXN00000000	Timme et al. 2013
Muenchen	baa1594	AOYV00000000	Timme et al. 2013
	baa1674	AOYT00000000	Timme et al. 2013
	SMu02	MATG00000000	Direct submission
	str. 08-1125	ARYX00000000	Direct submission
	str. 2009K-0951	ARYW00000000	Direct submission
	ATCC 51959	AOZC00000000	Timme et al. 2013
Indiana Braenderup	ATCC BAA-664	AOZM00000000	Timme et al. 2013
	CFSAN000756	APAP00000000	Timme et al. 2013
	str.3114	HF969015	Bronowski et al. 2013
Bovismorbificans	CVM N45400	LHGE00000000	Direct submission
Litchfield	CFSAN001076	APAN00000000	Timme et al. 2013
	C500	CP007639	Li et al. 2014
	ATCC10708	CP012344	Yao et al. 2016
	SC-B67	AE017220	Chiu et al. 2005
Paratyphi C Virchow	str. 0006	AOZL00000000	Timme et al. 2013
	RKS4594	CP000857	Liu et al. 2009
	SL491	ABFH00000000	Fricke et al. 2011
	ATCC51955	AOXL00000000	Timme et al. 2013
Paratyphi B var. Java Schwarzengrund	NCTC5706	LT571437	Direct submission
	CVM19633	CP001127	Fricke et al. 2011
	SL480	ABEJ00000000	Fricke et al. 2011
	CVM N47716	LHJK00000000	Direct submission
Brandenburg	CVM N40367	JYYG00000000	Direct submission
	CVM N45949	LHHJ00000000	Direct submission
	CVM N51253	LHMR00000000	Direct submission
	27027	SRR1965225	Ashton et al. 2016
Sandiego	60139	SRR1968628	Ashton et al. 2016
	CFSAN001084	NZ_APAF00000000	Timme et al. 2013
Eastbourne	ATCC BAA-1673	AOYK00000000	Timme et al. 2013
Poona	420	AOYW00000000	Timme et al. 2013
Muenster	315	AOYX00000000	Timme et al. 2013

Other *Salmonella* spp.

<i>S. enterica</i> subsp. <i>arizonae</i>	RKS2983	CP006693.1	Direct submission
<i>S. enterica</i> subsp. <i>houtenae</i>	ATCC BAA-1581	CM001471.1	Direct submission
<i>S. enterica</i> subsp. <i>salamae</i>	1369-73	GCA_001448865.1	Yoshida et al. 2016
<i>S. enterica</i> subsp. <i>diarizonae</i>	11-01853	CP011289.1	Direct submission
<i>S. enterica</i> subsp. <i>indica</i>	1121	GCA_000486225.1	Timme et al. 2013
<i>S. bongori</i>	NCTC12419	NC_015761.1	Fookes et al. 2011

Draft

Primer set	Dispensation
fliD-ST_seq1	TGACTGATCTAGCTCGCTATGACAGCACACTATACGCATCA
fliD-ST_seq2	AGCATACGACTCACTGACGAGAGTGCACGACGCGATCGT
sopE2_seq1	TATATCGACAGTCATCTCTCTAGTAGCTCG
sopE2_seq2	GATGACGTATACTG
spaO_seq1	TCAGCGTCTGCAGTGCGCTAGC
spaO_seq2	GTGATCACGCG

Draft