RESEARCH LETTER

Development of a PCR test system for specific detection of Salmonella Paratyphi B in foods

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Salmonella Paratyphi B; multiplex PCR; comparative genomics; detection.

Abstract
Salmonella enterica serotype Paratyphi B is a globally distributed human-specific pathogen causing paratyphoid fever. The aim of this study was to develop a rapid and reliable polymerase chain reaction (PCR) assay for its detection in food. The SPAB_01124 gene was found to be unique to S. Paratyphi B using comparative genomics. Primers for fragments of the SPAB_01124 gene and the Salmonella-specific invA gene were used in combination to establish a multiplex PCR assay that showed 100% specificity across 45 Salmonella strains (representing 34 serotypes) and 18 non-Salmonella strains. The detection limit was 2.2 CFU mL⁻¹ of S. Paratyphi B after 12-h enrichment in pure culture. It was shown that co-culture with S. Typhimurium or Escherichia coli up to concentrations of 3.6 × 10⁵ CFU and 3.3 × 10⁴ CFU, respectively, did not interfere with PCR detection of S. Paratyphi B. In artificially contaminated milk, the assay could detect as few as 62 CFU mL⁻¹ after 8 h of enrichment. In conclusion, comparative genomics was found to be an efficient approach to the mining of pathogen-specific target genes, and the PCR assay that was developed from this provided a rapid, specific, and sensitive method for detection of S. Paratyphi B.

Introduction
Salmonella enterica serotype Paratyphi B is a major foodborne pathogen worldwide, and it is the most significant of any Salmonella serotype in this regard (Desenclos et al., 1996). It is a highly adapted, human-specific pathogen that causes self-limited gastroenteritis and paratyphoid fever (enteric fever). In recent times, paratyphoid has been considered a more serious problem than typhoid in many developing regions (Andrews-Polymenis et al., 2010). Infection can occur via human-to-human transmission, but contaminated food, such as meat, milk, and poultry products, is another prominent cause (Miko et al., 2002; Prager et al., 2003). Therefore, the effective detection of S. Paratyphi B in food is key to reducing infection rates.

Traditionally, the detection of Salmonella mostly relied on microbiological methods and serological tests that were important for epidemiological surveys. It entailed classifying Salmonella into 50 serogroups based on somatic antigens (O-antigens), and these were further divided into more than 2500 serotypes according to their flagella (H-antigens) (Fitzgerald et al., 2007). However, the traditional serotyping method is labor intensive, expensive, complicated, and time-consuming (Kim et al., 2006). Furthermore, it does not allow for partial serotyping owing to the lack of standardization across antisera and incorrect typing due to loss of surface or flagellar antigens (Yoshida et al., 2007; Woods et al., 2008). On account of lower specificity and sensitivity, the method is unable to identify some Salmonella serotypes. Thus, it has become imperative that more sensitive, accurate, and rapid detection methods are developed.

Recently, a number of molecular methods have been used for Salmonella detection and serotyping; these have been based on genotypic characteristics rather than less stable phenotypic features (Kim et al., 2006; Arrach et al., 2008). The polymerase chain reaction (PCR) is one of these methods, and it has been shown to be more powerful than traditional approaches to microbial detection (Kim et al., 2006; Fitzgerald et al., 2007; Silva et al.,...
2011). After identifying the invA gene as unique to Salmonella, Rahn et al. (1992) showed that this genus could be identified by PCR using a pair of specific primers. PCR has also been used for distinguishing of Salmonella serotypes; for example, the sdfI, ViaB, and Spy genes were used to identify S. Enteritidis, S. Typhi, and S. Typhimurium, respectively (de Freitas et al., 2010).

In earlier studies on Salmonella spp. serotyping by PCR, the detection indicators were usually serotype-specific genes, such as those for H-antigen, O-antigen, Vi-capsular antigen, and 16S rRNA. Recently, identification by means of detecting sequence polymorphisms has become more common (Hirose et al., 2002; McQuiston et al., 2004). However, many of these established multiplex PCR assays (of both varieties) struggle to reach the sensitivity required for testing in the food industry and epidemiology. Therefore, it is important that we find new serotype-specific targets and develop more efficient molecular methods for their detection. With the increasing application of bioinformatics technology and comparative genomics, more and more new serotype-specific targets, such as STM4495, SEN1392, SCH_0971, SeD_A1226, and SGI033, are being discovered (Akiba et al., 2011; Liu et al., 2012).

The fliC gene, which encodes flagellin antigens, is currently used for identification of S. Paratyphi B, but it does not accurately distinguish between Paratyphi B and other Salmonella serotypes (Yang et al., 2012). Therefore, the objective of this study was (1) to mine novel serotype-specific targets for detection of S. Paratyphi B using a comparative genomic approach; (2) to establish a rapid and accurate PCR assay targeting this gene; and (3) to evaluate this assay for detection of S. Paratyphi B.

Materials and methods

Bacterial strains

A total of 45 Salmonella strains and 18 non-Salmonella foodborne pathogens (Table 1) were acquired from the China Center of Industrial Culture Collection (CICC), the National center for medical culture collections (CMCC), and the American Type Culture Collection (ATCC). The Salmonella strains were stored in Luria–Bertani (LB) medium containing 25% glycerol at −70 °C.

Genomic DNA extraction

Genomic DNA of the bacteria from enrichment cultures was isolated using a bacterial genomic DNA extraction kit (China). The extracted DNA was validated by PCR using primers 139–141 (Table 2) and stored at −20 °C until their use as templates for the PCR reaction.

Screening for S. Paratyphi B-specific genes

The genomic sequence of S. Paratyphi B strain SPB7 (NC_010102.1) was downloaded from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). Using the methods of comparative genomics, we screened for DNA fragments specific to S. Paratyphi B (Liu et al., 2011). Each of the genomic sequences (on the size of the gene sequence itself) was BLASTed (basic local alignment search tool) against the database of genomic DNA, including the genome sequence of Salmonella and non-Salmonella. When the fragments were matched with BLAST outputs with E-values higher than 0.01, these were considered as specific targets for S. Paratyphi B detection and used as candidate detection fragments for PCR assay. The genome sequences did not only exhibit low homology with non-Salmonella but also high homology with S. Paratyphi B.

Primer design for PCR

Primers targeting the selected candidate gene(s) were designed using Primer PREMIER 5.0 software (PREMIER Biosoft International, Palo Alto, CA) and synthesized by Sangon Biotech (Shanghai, China). The two primer pairs are shown in Table 2. The specificity of the primers was verified using BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Using online bioinformatic tools (http://insilico.ehu.es/PCR/index.php?mo=Salmonella), in silico PCR amplification was carried out to further analyze the specificities of the gene amplification.

Multiplex PCR for detection of Salmonella spp. and S. Paratyphi B

PCR was used to amplify the Salmonella and S. Paratyphi B marker genes that were each targeted by a pair of primers (forward and reverse). Each 25 μL PCR reaction mixture contained 2.5 μL of 10× buffer (75 mmol Tris-Cl, 20 mmol (NH4)2SO4, 0.01% (v/v) Tween-20, 4.0 μL of MgCl2 (25 mmol), 1.5 μL of dNTP (2.5 mmol), 0.5 μL of Taq polymerase (5 units μL−1), and 2 μL of pPBM23 primers (10 μmol), 0.5 μL of 139–141 primers (10 μmol), and 5 μL of template DNA (134 mkg mL−1). The PCR reaction procedure consisted of an initial denaturation step of heating at 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s. Then there was a final extension step of 72 °C for 10 min. The products of the PCR reaction were observed under ultraviolet light after electrophoretic separation on 2% agarose gel in TAE buffer [40 mM Tris-acetate (pH 8.0), 1 mM EDTA] and staining with ethidium bromide.
<table>
<thead>
<tr>
<th>Strain (Salmonella)</th>
<th>Source</th>
<th>Number</th>
<th>Result</th>
<th>Strain (Salmonella)</th>
<th>Source</th>
<th>Number</th>
<th>Result</th>
<th>Strain (non-Salmonella)</th>
<th>Source</th>
<th>Number</th>
<th>Result</th>
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<td>S. Kentucky</td>
<td>CICC21488</td>
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<td>Escherichia coli</td>
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<td>S. Bazenheid</td>
<td>CICC21587</td>
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<td>–</td>
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<td>CICC21527</td>
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<td>–</td>
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<td>Enterococcus avium</td>
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<td>–</td>
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<td>S. Dublin</td>
<td>CMCC50761</td>
<td>1</td>
<td>–</td>
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<td>S. Miam</td>
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<td>–</td>
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<td>CICC21508</td>
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<td>S. Anatum</td>
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<td>1</td>
<td>–</td>
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<td>CICC21671</td>
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<td>S. Meagutis</td>
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<tr>
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<td>–</td>
<td>S. London*</td>
<td>CICC21502</td>
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<td>S. Aberdeen</td>
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<td>S. Blockley</td>
<td>CICC21489</td>
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<td>–</td>
<td>S. Adelaide</td>
<td>CICC21505</td>
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<tr>
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<td>–</td>
<td>S. Wandswerth</td>
<td>CICC21504</td>
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<tr>
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<td>–</td>
<td>S. Dakar</td>
<td>CICC21507</td>
<td>1</td>
<td>–</td>
<td></td>
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<tr>
<td>S. Bovismorbificans</td>
<td>CICC21499</td>
<td>1</td>
<td>–</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

+, positive result; –, negative result.

*Laboratory-stored strain.
Salmonella invA Paratyphi B S. (GB4789.4-2010). Inoculum of artificially contaminated milk was achieved by inoculating S. cultured with S. and Salmonella Typhimurium (ATCC35150), and nonselective enriched sterile saline solution. The suspensions contained microorganisms were serially diluted in 10-fold steps in 9 S. Typhimurium 10^2 CFU of the same serogroup, and Paratyphi B strains. The results confirmed that they are corresponding to the correct target gene.

**Specificity and sensitivity of the PCR assay**

The specificity of the PCR assay was verified using the DNA of 45 Salmonella strains and 18 non-Salmonella strains (Table 1).

To test the sensitivity of the PCR assay, S. Paratyphi B was cultured overnight and then serially diluted in sterile saline in 10-fold steps down to 1 : 10^7, and 1 mL of each dilution was inoculated into an equal volume of fresh LB culture medium at 37 °C for 12 h. After this enrichment step, 1 mL of each dilution was used for DNA extraction via a bacterial genomic DNA extraction kit. PCR was used to amplify the targeted S. Paratyphi B marker genes, and the products were separated and detected as in section 2.5. The number of colony-forming unit(s) (CFU) of each dilution was determined by a standard plate count.

**Evaluation of interference**

To test whether the PCR assay was subject to significant interference from other bacteria, S. Paratyphi B was co-cultured with S. Typhimurium (CMCC51005) or Escherichia coli (ATCC35150), and nonselective enriched Salmonella, containing other non-S. Paratyphi B strains. Salmonella Typhimurium and S. Paratyphi B belong to the same serogroup, and E. coli often accompany Salmonella spp. in contaminated raw food.

Eight hour cultures of these potentially interfering microorganisms were serially diluted in 10-fold steps in sterile saline solution. The suspensions contained 3.3–33 000 CFU of E. coli and 360–3,6 × 10^6 CFU of S. Typhimurium. One milliliter of each dilution was inoculated into LB culture medium containing 2.2 × 10^5 CFU of S. Paratyphi B at 37 °C for 12 h. Following DNA extraction, the PCR assay was carried out.

**Artificially contaminated milk sample**

Fresh milk from the local supermarket was determined to be free of S. Paratyphi B by standard methods (GB4789.4-2010). Inoculum of S. Paratyphi B was serially diluted 10-fold with sterile saline solution. Preparation of artificially contaminated milk was achieved by inoculating 1 mL of diluted inoculum (6.2–62 000 CFU) into 10 mL of milk (Silva et al., 2011), which was transferred to LB culture medium at 37 °C for 6, 8, 10, and 12 h. Finally, the templates extracted using the bacterial genomic DNA extraction kits were prepared for PCR. The number of CFU in the initial inoculum was found using a standard count.

**Results**

**Comparative genomics for screening of S. Paratyphi B-specific target genes**

The genomic sequences of S. Paratyphi B were compared with the genomic sequences in the NCBI databanks using the BLASTN program. From this, we found 16 genomic fragments that were expected to exist only in S. Paratyphi B and these were matched with BLASTN outputs with expect (E) values higher than 0.01. Accordingly, 26 primer sets were designed for specific amplification of these 16 genomic fragments. Subsequently, the specificity of the primer sets was validated in 45 Salmonella strains (34 serotypes) and 18 non-Salmonella (data not shown). The results showed that only the pPBm23 primers designed for the SPAB_01124 gene were 100% specific for S. Paratyphi B. The 25 primers for other 15 genomic sequences were not serotype-specific fragments due to obtaining of positive results in the other Salmonella serotypes.

**Design of PCR for S. Paratyphi B**

The pPBm23 (forward and reverse) primers were used for amplification of a 384 bp DNA sequence of SPAB_01124, while a 284 bp sequence of the traditional Salmonella-specific detection gene, invA, was amplified via the primers, 139–141, and used as a control. Based on these two target DNA sequences, a duplex PCR assay for S. Paratyphi B detection was established (Fig. 1). The 384 bp products were recovered from the gel and sequenced. The results confirmed that they are corresponding to the correct target gene.

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The specificity of the PCR assay was evaluated in 45 Salmonella and 18 non-Salmonella strains. The results revealed that only S. Paratyphi B produced the two expected bands for the Salmonella- and Paratyphi B-specific sequences of invA and SPAB_01124, respectively (Table 1). The other 33 Salmonella serotypes only showed the band for invA, identifying them as being of the

<table>
<thead>
<tr>
<th>Target organism</th>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
<th>PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. Paratyphi B</td>
<td>SPAB_01124</td>
<td>pPB23</td>
<td>atatatgtctttgcgtgccttc</td>
<td>384</td>
</tr>
<tr>
<td>Salmonella</td>
<td>invA</td>
<td>139–141</td>
<td>gtaaatatgaccagtgrrg</td>
<td>284</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>tcatgcaccgtcaggaacc</td>
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</tr>
</tbody>
</table>

---

**Table 2. Primer sets for multiplex PCR detection of S. Paratyphi B**

The specificity and sensitivity of PCR

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Salmonella genus (Table 1), whereas the 16 non-Salmonella strains did not produce any bands.

The detection limit for pure cultures of S. Paratyphi B was c. 2.2 CFU mL\(^{-1}\) after 12 h enrichment at 37 °C in LB medium (Fig. 2).

**Evaluation of interference test**

This test was designed to investigate whether genomic DNA of other Salmonella serotypes might interfere with multiplex PCR detection of the target organism (Daniell et al., 2012).

The PCR assay indicated that all treatments with different concentrations of non-S. Paratyphi B strains yielded positive results for the Salmonella-specific invA sequence. Positive results were found for S. Paratyphi B (2.2 \(\times\) 10\(^2\) CFU) in the presence of up to 3.6 \(\times\) 10\(^5\) CFU of S. Typhimurium or up to 3.3 \(\times\) 10\(^4\) CFU of E. coli, respectively (Fig. 3).

**Detection in artificially contaminated milk**

Milk samples spiked with five concentrations (10-fold intervals) of S. Paratyphi B from 6.2 to 62 000 CFU mL\(^{-1}\) were subjected to the PCR assay after 6, 8, 10, and 12 h of enrichment (Table 3). The target amplification products were detected for all concentrations after at least 10 h of enrichment. After 8 h of enrichment, the assay could detect up to 6.2 \(\times\) 10\(^2\) CFU mL\(^{-1}\) of S. Paratyphi B and after 6 h up to 6.2 \(\times\) 10\(^3\) CFU mL\(^{-1}\) gave a positive result.
Discussion

There is an urgent need to establish reliable high-resolution serotyping methods for Salmonella-related foodborne outbreaks. The aim of this study was to develop such a method for detecting S. Paratyphi B by PCR. The comparative genomic method is a successful approach to identify serotype-specific targets such as S. Enteritidis, S. Typhimurium, and S. Typhi (Banavandi et al., 2005; Liu et al., 2012). The approach only relies on collecting information of genomic sequences of Salmonella serovars, analyzing genomic information by BLAST system and validating the results by PCR (Liu et al., 2012).

The SPAB_01124 gene encodes a hypothetical protein that may be a dinucleotide-utilizing enzyme involved in molybdopterin and thiamine biosynthesis. In our study, this gene was found to be unique to S. Paratyphi B. After using comparative genomic analysis to identify 16 potential target DNA fragments for the exclusive detection of S. Paratyphi B, the primer set for SPAB_01124 was found via simulated PCR to successfully discriminate between S. Paratyphi B and other serotype Salmonella strains as well as non-Salmonella strains.

Based on a pair of pPBm23 primers targeting the SPAB_01124 gene and a pair of 139–141 primers targeting the invA gene that is specific to the Salmonella genus (Rahn et al., 1992), a novel duplex PCR assay was developed. The invA gene has been applied in the validation and standardization of PCR for Salmonella detection in food in Europe (Hoorfar, 1999); thus, using this gene in our PCR assay allowed us to effectively distinguish Salmonella from other non-Salmonella bacteria, even in the complex microbial environments of food.

Salmonella Paratyphi B identification by PCR detection of the fliC gene is an approach commonly used at present; however, it may yield false positives as the gene also occurs in the common serotypes, typhimurium and Kentucky, and in less common serotypes such as Aberdeen, Bergen, and Kedougou (O’Regan et al., 2008; Yang et al., 2012). Our assay showed 100% specificity across the 45 Salmonella strains (representing 34 serotypes) and 18 non-Salmonella strains that were tested; these included the Typhimurium, Kentucky, and Aberdeen serotypes.

This indicates that our assay is superior to the fliC-based method in terms of specificity. Yang et al. (2012) have reported on the use of fliC for PCR detection of S. Paratyphi B, and their method comprised a nonselective enrichment step and a total DNA extraction procedure. Enrichment over 12 h was required to provide a sufficient number of viable cells and to effectively dilute inhibitory substances present in the food matrices. It has been shown that the PCR is affected by these substances, which include proteases, DNase, polysaccharides, fats, calcium salts, and phenolic compounds (Silva et al., 2011). As the number of dead cells contained in the samples does not increase, the false-positive results from the dead cells could be effectively avoided. In this study, the detection limit after 12 h of enrichment was c. 2.2 CFU mL⁻¹, which is substantially lower than the 3.7 × 10² CFU mL⁻¹ reported by Yang et al. (2012). In our work, a bacterial genomic DNA extraction kit was used to extract the total DNA of samples. Although the method was more costly and time-consuming than the boiling technique (Kumar et al., 2006), it was more efficient at harvesting genomic DNA and reducing interference by PCR inhibitors.

The reliability and selectivity of PCR detection of S. Paratyphi B was evaluated via an interference test and an artificially contaminated milk test. The results showed that at least 10⁴ CFU mL⁻¹ of S. Typhimurium or E. coli was needed to interfere with identification of S. Paratyphi B: this is much higher than the 5 × 10² CFU mL⁻¹ reported by Villamizar et al. (2008), suggesting that our assay is more robust in the face of interfering microorganisms. The detection limit of our PCR assay was c. 2.2 CFU mL⁻¹, and this makes it more sensitive than that of Yang et al. (2012) who reported a limit of 3.7 × 10¹ CFU mL⁻¹ for S. Paratyphi B in artificially contaminated food. The sensitivity and specificity of our PCR assay was further confirmed by the artificially contaminated milk test, which indicated that it could detect as little as 62 CFU mL⁻¹ of S. Paratyphi B in milk samples after 8 h of enrichment.

Conclusions

Using a comparative genomics methodology, SPAB_01124 was found to be a novel gene target for the detection of S. Paratyphi B. The result shows that this comparative genomics method is an effective tool for mining new Salmonella serotype-specific genes. Based on the SPAB_01124 gene, we established an efficient PCR assay for highly specific and sensitive detection of S. Paratyphi B. These results suggest that this PCR assay will make a useful serotyping technique for identification of S. Paratyphi B in food.

### Table 3. Salmonella Paratyphi B detection results for artificially contaminated milk samples

<table>
<thead>
<tr>
<th>Milk sample (CFU)</th>
<th>6 h</th>
<th>8 h</th>
<th>10 h</th>
<th>12 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. Paratyphi</td>
<td>6.2 × 10⁰</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>CICC21495</td>
<td>6.2 × 10ⁱ</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>6.2 × 10²</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>6.2 × 10³</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>6.2 × 10⁴</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

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