Molecular Phylogeny of Salmonellae: Relationships among Salmonella Species Determined from gyrA, gyrB, parC, and parE Genes

CHARIS AMARANTINI* AND DHIRA SATWIKA

Faculty of Biotechnology, Duta Wacana Christian University
Jalan dr. Wahidin Sudirohusodo 5-19, Yogyakarta 55224, Indonesia

Study on molecular characteristics of Salmonella from clinical isolates was done in order to find out its relationship, especially those isolated from Indonesia. Partial sequence of genes belonging to QRDR region, i.e. gyrA, gyrB, parC, and parE were employed. Specific primer pairs covering those genes are used to amplify the bacterial DNA obtained. The amplicons were then analyzed by means of sequencing, and the sequences are analysed bioinformatically to find out similarities and build phylogenetic trees. By comparing all of the phylogenetic tree from QRDR region, this study revealed gyrA as the most suitable gene for rapidly identify member of salmonellae as it gives better separation of samples being analysed. However, the use of parC is recommended as it gives a consistent and reliable value to separate member of Salmonella and other Enterobacter. Further studies are under way to include member of this group, like E. coli, and the use of full sequence of QRDR genes region to verify this report.

Key words: gyrA, parC, QRDR, Salmonella

Salmonellae are a diverse group of gram-negative bacteria and consist of a number closely related organisms belonging to the family Enterobacteriaceae. For a long time, the taxonomic classification and nomenclature of this group changed several times. The last classification systems have been validly proposed for two-species systems, Salmonella enterica and Salmonella bongori (Chang et al. 1997). Currently, Shelobolina et al. (2004) discovered Salmonella subterranea sp. nov. as a new species belongs to the genus Salmonella.

In addition to the taxonomic classification of two-species systems, the salmonellae were categorized by serotype. There are over 2,500 serotypes associated with gastroenteritis and typhoid fever in human. The majority of these serotypes are often extremely difficult to be separated based on its biochemical characteristics. Therefore, it would be useful to classify these groups based on molecular typing methods.

Molecular phylogenetic approach for classification of salmonellae is important due to the increased spread of Salmonella strains, especially Salmonella enterica serovar Typhi (S. Typhi) with fluoroquinolone reduced susceptibility. Reports already available mentioning the use of genetic traits to differentiate this bacteria (for example: Tajbakhsh et al. 2011, Muthu et al. 2014). Nalidixic acid resistant of S. Typhi (NARST) strains with decreased susceptibility to ciprofloxacin (0.125-1 µg/mL) becomes a major problem in the Indian subcontinent (Capoor et al. 2007), and molecular analysis showed mutations of
some genes in quinolone resistance-determining region (QRDR). It was reported, a point mutation in QRDR of the gyrA gene at various sites, especially at codons coding for serine at position 83 and aspartate at position 87 (Afzal et al. 2013). Other mutation was observed in the genes coding for DNA gyrase (gyrA or gyrB) or topoisomerase (parC and parE) in the resistant strain (Muthu et al. 2014).

It was reported earlier some S. Typhi strains are resistant to nalidixic acid. The taxonomic classification of these strains based on 16S rRNA gene showed the sensitive and resistant isolates can not be separated into different clades (Amarantini and Budiarsro 2013). Detailed analysis for bacterial phylogenetic relationships through the QRDR of the gyrA and gyrB subunits of DNA gyrase and the parC and parE subunits of topoisomerase IV might be possible for a better classification than 16S rRNA regions. The present study was done to assess the use of gyrA and gyrB genes in QRDR and parC and parE genes in topoisomerase IV for determining the phylogenetic relationship among sensitive and resistant S. Typhi isolates.

MATERIALS AND METHODS

Bacterial Strains. Four isolates used in this study were obtained from the previous research (Amarantini and Budiarsro 2013); i.e. two nalidixic acid-resistant and two nalidixic acid-sensitive isolates. Two isolates from reference collection of PT Biofarma (S. Typhi NCTC 786) and BLK Yogyakarta (S. Typhi O) were also included.

DNA Isolation. All isolates were cultured in Brain Heart Infusion Broth at 37 °C for 18 h prior to DNA extraction. To isolate chromosomal DNA, 1 ml of overnight culture were put into a 1.5 mL centrifuge tube and were centrifuged at 5000 rpm, 15 min to obtain the cells. Isolation of DNA was carried out in accordance with the phenol-chloroform-isoamyl alcohol method (Sambrook et al. 1989).

PCR Experiments of QRDRs of gyrA, gyrB, parC, and parE Genes of S. Typhi. DNA isolated from bacterial strains were amplified by PCR using specific primer (Table 1) for gyrA, gyrB, parC, and parE (Ling et al. 2003). The PCR was performed using DreamTaq™ Green PCR Master Mix in total reaction volume of 50µL containing 25µL of 2x DreamTaq™ Green PCR Master Mix, 1 µL of 1 µM primer stocks, and 1 µL of template DNA.

The PCR reaction mixtures were amplified for 35 cycles with initial-denaturation at 95 °C for 5 min, denaturation at 94°C for 1 min, annealing at 52 °C for 1 min, and extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min. An aliquot of 5 µL of each amplified product was electrophoresed in 1.2% (wt/vol) agarose gel using 1x TBE buffer gel stained with Sybr® Safe DNA stain (Life technologies). A 100-bp DNA ladder (Fermentas, Germany) were included as molecular weight marker.

DNA Sequencing and Phylogenetic Analysis. PCR products were purified and sequenced by outsourcing the samples to Macrogen Inc, Korea. The nucleotide sequences were edited and assembled using SeqMan and EditSeq (DNA Star, Laser Gene 6, Madison, WI, USA). Phylogenetic tree was constructed with Mega v5 (Tamura et al. 2011) with the neighbor-joining algorithm (Saito and Nei 1987). The evolutionary distance matrix for the neighbor-joining method was generated in accordance with the description introduced by Jukes and Cantor (1969). The matrix of the nucleotide similarity and difference was generated with PHYDIT software (Chun 1999).

RESULTS

We have amplified and sequenced two DNA fragments of S. Typhi containing gyrA and gyrB QRDRs. We also used two pairs of primers to amplify

Table 1 Primers used to sequence gyrB/gyrA and parE/C (Ling et al. 2003)

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gyrA</td>
<td>F, 5’-TgTCCGAGATGGCCTGAAGC-3’</td>
<td>347</td>
</tr>
<tr>
<td></td>
<td>R, 5’-TACCCTAGATTTATCCACG-3’</td>
<td></td>
</tr>
<tr>
<td>gyrB</td>
<td>F, 5’-CAAACCTGCGGCACTGACGG-3’</td>
<td>345</td>
</tr>
<tr>
<td></td>
<td>R, 5’-TTCCGGATCATGACGATAGA-3’</td>
<td></td>
</tr>
<tr>
<td>parC</td>
<td>F, 5’-ATGACCGCATATGGCAGAGCGG-3’</td>
<td>412</td>
</tr>
<tr>
<td></td>
<td>R, 5’-TGACCGAGTCTCGCTTAACAG-3’</td>
<td></td>
</tr>
<tr>
<td>parE</td>
<td>F, 5’-GACCAGCTGTTTTCCTGTTG-3’</td>
<td>272</td>
</tr>
<tr>
<td></td>
<td>R, 5’-AGCAGAGTAGCGATATGCAA-3’</td>
<td></td>
</tr>
</tbody>
</table>
the parC and parE QRDRs. As expected, two amplification products of 435 and 297 bp were obtained and their nucleotide sequences were determined. From these sequence data, we construct the phylogenetic structure and determine the genetic relationship among the nalidixic acid-sensitive and -resistant isolates.

As shown in Figure 1, the phylogenetic analysis determined by gyrA sequence clearly separate the sensitive and resistant isolates into different clusters. Two resistant isolates (BPE 127.1 MC and BPE 122.4 CCA) were delineated using the gyrA gene. The percentage similarity within each strains ranged from 73.25-93.35% (Table 2). In contrast to the gyrA dendogram, the phylogenetic structure of Salmonella on the basis of gyrB gene sequences (345 bp) were not able to distinguish the sensitive and resistant isolates (Fig 2). The resistant isolates were grouped as the same cluster with the sensitive isolates. Unlike the gyrA dendogram, the similarity percentage between each

![Fig 1 Phylogenetic tree of Salmonella strains based on gyrA sequences analysis. The tree was constructed by neighbor-joining method. Bar, 1 substitution per 10 nucleotides.](image)

**Table 2** Nucleotide similarity values (%) and the number of nucleotide differences between Salmonella strains based on gyrA sequence

<table>
<thead>
<tr>
<th></th>
<th>S. Typhi O (BLK Yogyakarta)</th>
<th>S. Typhi BPE 122.1 CCA</th>
<th>S. Typhi BPE 122.4 CCA R*</th>
<th>S. Typhi BPE 127.1 MC R*</th>
<th>S. Typhi NCTC 786</th>
<th>S. Typhi RSK 5.1 SSA</th>
<th>HQ176366.1</th>
<th>HQ176368.1</th>
<th>KC121321.1</th>
<th>AY323807.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. Typhi BPE 127.1 MC R*</td>
<td>91.25</td>
<td>89.30</td>
<td>93.02</td>
<td>---</td>
<td>25/328</td>
<td>51/359</td>
<td>194/348</td>
<td>194/348</td>
<td>153/283</td>
<td>134/232</td>
</tr>
<tr>
<td>S. Typhi RSK 5.1 SSA</td>
<td>88.52</td>
<td>89.85</td>
<td>90.27</td>
<td>92.38</td>
<td>---</td>
<td>39/330</td>
<td>178/324</td>
<td>178/324</td>
<td>154/282</td>
<td>131/228</td>
</tr>
<tr>
<td>HQ176366.1</td>
<td>42.69</td>
<td>41.13</td>
<td>43.26</td>
<td>44.25</td>
<td>45.06</td>
<td>38.64</td>
<td>---</td>
<td>0/434</td>
<td>3/290</td>
<td>16/238</td>
</tr>
<tr>
<td>HQ176368.1</td>
<td>42.69</td>
<td>41.13</td>
<td>43.26</td>
<td>44.25</td>
<td>45.06</td>
<td>38.64</td>
<td>100.00</td>
<td>---</td>
<td>3/290</td>
<td>16/238</td>
</tr>
<tr>
<td>KC121321.1</td>
<td>44.37</td>
<td>45.42</td>
<td>44.88</td>
<td>45.94</td>
<td>45.39</td>
<td>45.77</td>
<td>98.97</td>
<td>98.97</td>
<td>---</td>
<td>18/235</td>
</tr>
<tr>
<td>AY323807.1</td>
<td>41.38</td>
<td>43.10</td>
<td>41.81</td>
<td>42.24</td>
<td>42.54</td>
<td>43.10</td>
<td>93.28</td>
<td>93.28</td>
<td>92.34</td>
<td>---</td>
</tr>
</tbody>
</table>
Fig 2  Phylogenetic tree of *Salmonella* strains based on gyrB sequences analysis. The tree was constructed by neighbor-joining method. Bar, 1 substitution per 10 nucleotides.

Table 3 Nucleotide similarity values (%) and the number of nucleotide differences between *Salmonella* strains based on gyrB sequence

<table>
<thead>
<tr>
<th>DQ447149.1</th>
<th>S. Typhi O (BLK Yogyakarta)</th>
<th>S. Typhi RSK 5.1 SSA</th>
<th>S. Typhi BPE 122.4 CCA R*</th>
<th>S. Typhi BPE 127.1 MC R*</th>
<th>S. Typhi NCTC 786</th>
<th>AY996867.1</th>
<th>AY370864.1</th>
<th>EF064853.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>94.59</td>
<td>95.13 (-)</td>
<td>93.36 (-)</td>
<td>94.08 (-)</td>
<td>94.37 (-)</td>
<td>69.42 (-)</td>
<td>43.50 (-)</td>
<td>38.69 (-)</td>
<td></td>
</tr>
<tr>
<td>16/296</td>
<td>0/267</td>
<td>20/301</td>
<td>17/287</td>
<td>233/762</td>
<td>278/492</td>
<td>65/284</td>
<td>43/92</td>
<td></td>
</tr>
<tr>
<td>S. Typhi O (BLK Yogyakarta)</td>
<td>S. Typhi RSK 5.1 SSA</td>
<td>S. Typhi BPE 122.4 CCA R*</td>
<td>S. Typhi BPE 127.1 MC R*</td>
<td>S. Typhi NCTC 786</td>
<td>AY996867.1</td>
<td>AY370864.1</td>
<td>EF064853.1</td>
<td></td>
</tr>
<tr>
<td>94.59</td>
<td>95.13 (-)</td>
<td>93.36 (-)</td>
<td>94.08 (-)</td>
<td>94.37 (-)</td>
<td>69.42 (-)</td>
<td>43.50 (-)</td>
<td>38.69 (-)</td>
<td></td>
</tr>
<tr>
<td>16/296</td>
<td>0/267</td>
<td>20/301</td>
<td>17/287</td>
<td>233/762</td>
<td>278/492</td>
<td>65/284</td>
<td>43/92</td>
<td></td>
</tr>
</tbody>
</table>

Fig 3  Phylogenetic tree of *Salmonella* strains based on parC sequences analysis. The tree was constructed by neighbor-joining method. Bar, 1 substitution per 10 nucleotides.
strains were nearly equal ranging from 98.94 - 100% (Table 3).

Figure 3 shows the phylogenetic structure of *Salmonella* based on parC gene sequences. The phylogenetic analysis determined by parC sequence give rise to a tree structure which clustered all the test strains into a cluster, with control strain S. Typhi O BLK separated into different cluster as a sole species. This finding is supported by the nucleotide similarity index value above 99.0% (Table 4).

An almost similar phylogenetic tree structure (Fig 4) was revealed when we analyzed the isolates based on parE gene sequences. All the tested isolates are separated into a cluster which also included some member of Enterobacter. However, a lower nucleotide similarity index was observed compared to the one produced which is based on parC gene sequences (Table 5). A lower bootstrap value was also observed, emphasizing the consistency of isolates separation based on parC gene.
DISCUSSION

DNA sequence analysis has become increasingly popular in determining the evolutionary relationships of bacteria (Tajbakhsh et al. 2011). In the present study we therefore determined the phylogenetic structure for six strains belonging to *S. Typhi* based on partial gyrA and gyrB sequences. The test strains comprised of two groups, resistant and susceptible to nalidixic acid.

Results of phylogenetic structure showed that all of the test strains were sharply separated, but the topology of the tree based on gyrA gene was very different to that of the gyrB. The phylogenetic tree based on gyrA gene showed that the resistant strains were organized into different clusters. Our data indicated that the gyrA nucleotide sequences showed much higher variations than gyrB. The maximum and minimum nucleotide similarity among *S. Typhi* strains based on gyrA sequence was 93.35% and 73.25%, respectively (Table 2). The phylogenetic tree based on gyrB gene grouped all six *S. Typhi* strains together in a single cluster (Fig 2). Data showed that the nucleotide sequences from these groups were very similar (Table 3) ranged from 98.94 - 100%. These facts indicated that they exhibited the closest relationship. Souza *et al.* (2011) observed that non-fluorine quinolones such as nalidixic acid may be sufficient to generate mutations that alter the susceptibility of *Salmonella* spp to fluoroquinolones. Mutations have rarely been reported in the gyrB gene (Ling *et al.* 2003). So, it is clear that the higher genetic variation in term of nucleotide similarity of gyrA in the test strains may be mainly due to mutations.

In general, the phylogenetic tree based on gyrA and gyrB genes showed the separation isolates into several clades with a better separation compared to the one based on 16S rRNA gene from the previous research (Amarantini and Budiarso 2013). The major difference is the position of the resistant isolates (BPE 122.4 CCA R* and BPE 127.1 MC R*) which clustered with the sensitive isolates in their analysis (Fig 5). The similarity values of 16S rRNA were higher than the gyrA and gyrB sequences (Table 6). They showed the closest 16S rRNA relatedness values (≥99.42 % similarity) among all of the test strains.

Comparison of gyrA and gyrB sequences and 16S rRNA sequences for phylogenetic analysis demonstrated that taxonomy based on 16S rRNA typing methods may not be enough for the delineation phylogenetic differences at the species level. The 16S

| Table 5 Nucleotide similarity values (%) and the number of nucleotide differences between *Salmonella* strains based on parE sequence |
|---|---|---|---|---|---|---|---|---|
| **Salmonella Typhi** | **Salmonella Typhi** | **Salmonella Typhi** | **Salmonella Typhi** | **Salmonella Typhi** | **Salmonella Typhi** | **Salmonella Typhi** | **Salmonella Typhi** |
| RSK 5.1 SSA | --- | 0/198 | 4/199 | 4/196 | 0/197 | 2/197 | 1/199 | 1/199 | 107/193 |
| BPE 127.1 MC R* | 100.00 | --- | 4/198 | 4/196 | 0/197 | 2/197 | 1/198 | 1/198 | 107/192 |
| NCTC 786 | 97.99 | 97.98 | --- | 0/200 | 4/197 | 6/200 | 4/207 | 4/207 | 113/203 |
| BPE 122.4 CCA R* | 97.96 | 97.96 | 100.00 | --- | 4/196 | 6/196 | 3/198 | 3/198 | 105/193 |
| BPE 122.1 CCA | 100.00 | 100.00 | 97.97 | 97.96 | --- | 2/196 | 1/197 | 1/197 | 106/191 |
| BPE 122.1 CCA | 98.98 | 98.98 | 97.00 | 96.94 | 98.98 | --- | 3/200 | 3/200 | 108/194 |
| (BLK Yogyakarta) | | | | | | | | | |
| Salmonella Typhi (AM283478.1) | 99.50 | 99.49 | 98.07 | 98.48 | 99.49 | 98.50 | --- | 1/481 | 134/233 |
| Salmonella Typhi (AB072701.2) | 99.50 | 99.49 | 98.07 | 98.48 | 99.49 | 98.50 | 99.79 | --- | 134/233 |
| Salmonella Typhi strain 35 (HQ698252.1) | 44.56 | 44.27 | 44.33 | 45.60 | 44.50 | 44.33 | 42.49 | 42.49 | --- |
Table 6  Nucleotide similarity values (%) and the number of nucleotide differences between Salmonella strains based on 16S rRNA

<table>
<thead>
<tr>
<th>S. Typhi BPE 122.4 CCA (R*)</th>
<th>S. Typhi BPE 127.1 MC (R*)</th>
<th>S. Typhi BPE 122.1</th>
<th>S. Typhi RSK 5.1 SSA</th>
<th>S. Typhi NCTC 786</th>
<th>Z47544</th>
<th>AF029227</th>
<th>X80724.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>---</td>
<td>3/1383</td>
<td>0/1381</td>
<td>2/1382</td>
<td>3/1381</td>
<td>5/1381</td>
<td>33/1377</td>
<td>45/1374</td>
</tr>
<tr>
<td>S. Typhi BPE 127.1 MC (R*)</td>
<td>99.78</td>
<td>---</td>
<td>3/1381</td>
<td>5/1382</td>
<td>6/1381</td>
<td>8/1381</td>
<td>36/1377</td>
</tr>
<tr>
<td>S. Typhi BPE 122.1</td>
<td>100.00</td>
<td>99.78</td>
<td>---</td>
<td>2/1381</td>
<td>3/1381</td>
<td>5/1381</td>
<td>33/1377</td>
</tr>
<tr>
<td>S. Typhi RSK 5.1 SSA</td>
<td>99.86</td>
<td>99.64</td>
<td>99.86</td>
<td>---</td>
<td>3/1381</td>
<td>5/1381</td>
<td>33/1377</td>
</tr>
<tr>
<td>S. Typhi NCTC 786</td>
<td>99.78</td>
<td>99.57</td>
<td>99.78</td>
<td>99.78</td>
<td>---</td>
<td>6/1381</td>
<td>34/1377</td>
</tr>
<tr>
<td>Z47544</td>
<td>99.64</td>
<td>99.42</td>
<td>99.64</td>
<td>99.64</td>
<td>99.57</td>
<td>---</td>
<td>36/1497</td>
</tr>
<tr>
<td>AF029227</td>
<td>97.60</td>
<td>97.39</td>
<td>97.60</td>
<td>97.60</td>
<td>97.53</td>
<td>97.60</td>
<td>---</td>
</tr>
<tr>
<td>X80724.1</td>
<td>96.72</td>
<td>96.51</td>
<td>96.72</td>
<td>96.72</td>
<td>96.65</td>
<td>96.68</td>
<td>97.30</td>
</tr>
</tbody>
</table>

rRNA genes couldn't be used as a suitable marker for classification of closely related bacterial species (Tajbakhsh et al. 2011). It was useful only for describing phylogenetic relationships between distantly related Enterobacteriaceae and can not be applied for intrageneric relationship (Dauga 2002).

In the present study, we also noted that the application of gyrA typing showed a better classification than gyrB. The gyrA gene exhibited high variation in nucleotide sequences (73.25 - 93.35% similarity). Because of this, the gyrA gene provided higher resolution than the gyrB gene. It is, therefore encouraging that gyrA was found to be the best marker for classification.

Based on these results, it is clear there are nucleotide polymorphisms occur among gyrA, gyrB, parC, and parE which result in defined clustering of the tested isolates. Nonetheless, it is recommended to employ parC as a preferred gene to distinguish Salmonella and its close relative, like member of Enterobacter, as it is sensitive and specific. A further study need to be done to ensure this assumption as full sequence of each QRDR genes were not included in this study.

ACKNOWLEDGMENTS

This research was funded by the Directorate General of Higher Education, Department of National Education (Hibah Fundamental) 2014, contract no:1348/K5/KL/2013 date 14-05-2014.

REFERENCES


Amarantini C, Budiarso TY. 2013. 16S rDNA typing of
Salmonellatyphi strains from different geographical locations in Sumba island East Nusa Tenggara