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

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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*

Penelitian ini dilakukan untuk mencari kekerabatan dan hubungan filogeni *Salmonella* yang berasal dari isolat klinis yang diisolasi dari Indonesia. Selama ini sudah dilaporkan pemanfaatan gen-gen yang berada pada daerah QRDR dapat digunakan sebagai penanda untuk menentukan hubungan kekerabatan tersebut. *Partial sequence* gen-gen pada daerah tersebut, yaitu gyrA, gyrB, parC dan parE digunakan untuk tujuan tersebut. Pasangan primer yang mengamplifikasi urutan parsial setiap gen digunakan dalam reaksi PCR, selanjutnya hasil amplifikasi DNA isolat sampel dianalisa dengan melakukan sekuensing. Setelah urutan parsial DNA setiap gen diketahui, dilakukan analisa secara bioinformatis dengan melakukan *alignment* DNA sampel dengan *database* yang tersedia. Analisa ini memberi data berupa indeks kesamaan nukleotida hingga pembuatan pohon filogeni, yang dapat digunakan untuk melacak dan mencari kekerabatan setiap isolat. Hasil penelitian menunjukkan bahwa gyrA merupakan gen yang dapat digunakan untuk identifikasi cepat anggota *Salmonella* karena memberi resolusi paling baik. Meskipun demikian, parC merupakan gen yang direkomendasikan untuk memisahkan anggota *Salmonella* maupun kelompok bakteri yang erat kekerabatannya, misal kelompok Enterobacter, karena konsistensi pemisahan yang baik.

Kata kunci : 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 fluor oquinol one 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). Nalidixicacid resistant of *S*. Typhi (NARST) strains with decreased susceptibility to ciprofloxacin (0.125-1 μgL^{-1}) becomes a major problem in the Indian subcontinent (Capoor *et al*. 2007), and molecular analysis showed mutations of

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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 Budiarso 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 Budiarso 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 centrifugetube 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 the gyrA, gyrB, parC, and parE Genes of *S.* Typhi. DNA isolated from bacterial strains were amplified by PCR using spesific 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 ºC for 5 min, denaturation at 94 ºC for 1 min, annealing at 52 ºC for 1 min, and extention at 72 ºC for 1 min, with a final extension at 72 ºC for 5 min. An aliquot of 5 µL of each amplified product was electrophorese 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)

Primer name	Primer sequence	Size (bp)
gyrA	F, 5'-TgTCCGAGATGGCCTGAAGC-3' R, 5'-TACCGTCATAGTTATCCACG-3'	347
gyrB	F, 5'-CAAACTGGCGGACTGTCAGG-3' R, 5'-TTCCGGCATCTGACGATAGA-3'	345
parC	F, 5' ATGAGCGATATGGCAGAGCG 3' R, 5' TGACCGAGTTCGCTTAACAG 3'	412
parE	F, 5' GACCGAGCTGTTCCTTGTGG 3' R, 5' AGCAGAGTAGCGATATGCAA 3'	272

the parC and parE QRDRs. As expected, two amplification products of 435 and 297 bp were obtanined 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

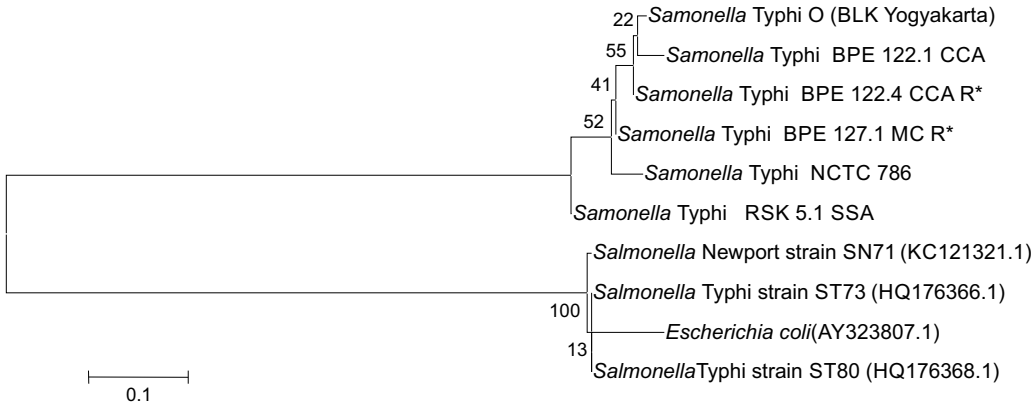

Fig 1 Phylogenetic tree of *Salmonella* strains based on gyrA sequences analysis. The tree was constructed by neighbor-joining method. Bar, 1 substitution per 10 nucleotides.

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

	S. Typhi O (BLK Yogyakarta)	*S.*Typhi BPE 122.1 CCA	*S.*Typhi BPE 122.4 CCA R*	*S.* Typhi BPE 127.1 MC R*	*S.* Typhi NCTC 786	*S.* Typhi RSK 5.1 SSA	HQ176368.1	HQ176366.1	KC121321.1	AY323807.1
S. Typhi O (BLK Yogyakarta)	---	25/340	23/346	30/343	38/331	49/350	192/335	192/335	158/284	136/232
*S.*Typhi BPE 122.1 CCA	92.65	---	41/363	38/355	33/325	126/471	249/423	249/423	155/284	132/232
*S.*Typhi BPE 122.4 CCA R*	93.35	88.71	---	25/358	32/329	67/370	202/356	202/356	156/283	135/232
S. Typhi BPE 127.1 MC R*	91.25	89.30	93.02	---	25/328	51/359	194/348	194/348	153/283	134/232
S. Typhi NCTC 786	88.52	89.85	90.27	92.38	---	39/330	178/324	178/324	154/282	131/228
S. Typhi RSK 5.1 SSA	86.00	73.25	81.89	85.79	88.18	---	262/427	262/427	154/284	132/232
HQ176368.1	42.69	41.13	43.26	44.25	45.06	38.64	---	0/434	3/290	16/238
HQ176366.1	42.69	41.13	43.26	44.25	45.06	38.64	100.00	---	3/290	16/238
KC121321.1	44.37	45.42	44.88	45.94	45.39	45.77	98.97	98.97	---	18/235
AY323807.1	41.38	43.10	41.81	42.24	42.54	43.10	93.28	93.28	92.34	---

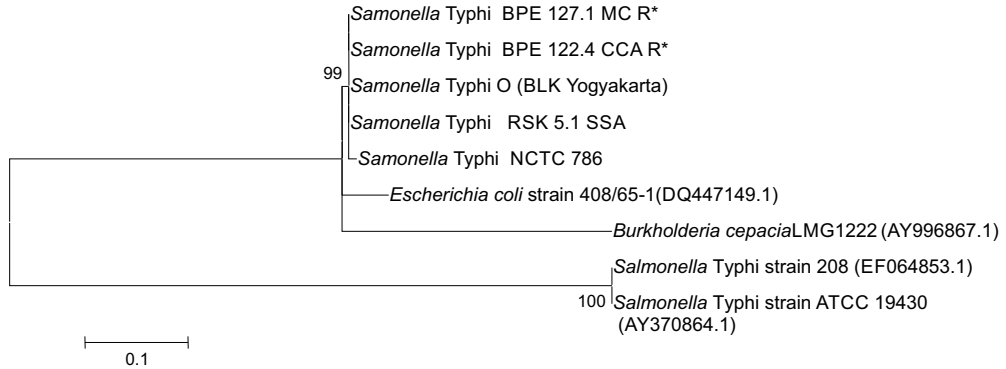

Fig 2 Phylogenetic tree of *Salmonella* strains based on gyrB sequences analysis. The tree was constructed by neighbor-joining method. Bar, 1 substitution per 10 nucleotides.

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

	DQ447149.1	*S.* Typhi O (BLK Yogyakarta)	*S.* Typhi RSK 5.1 SSA	*S.* Typhi BPE 122.4 CCA R*	*S.* Typhi BPE 127.1 MC R*	*S.* Typhi NCTC 786	AY996867.1	AY370864.1	EF064853.1
DQ447149.1	---	16/296	13/267	20/301	17/287	16/284	233/762	278/492	420/685
S. Typhi O (BLK Yogyakarta)	94.59	---	0/267	1/296	1/283	3/284	68/296	150/288	150/288
S. Typhi RSK 5.1 SSA	95.13	100.00	---	0/267	0/267	2/267	59/267	131/258	131/258
S. Typhi BPE 122.4 CCA R*	93.36	99.66	100.00	---	1/287	3/284	71/301	152/292	152/292
S. Typhi BPE 127.1 MC R*	94.08	99.65	100.00	99.65	---	2/284	66/287	145/278	145/278
S. Typhi NCTC 786	94.37	98.94	99.25	98.94	99.30	---	65/284	145/277	145/277
AY996867.1	69.42	77.03	77.90	76.41	77.00	77.11	---	297/492	765/1156
AY370864.1	43.50	47.92	49.22	47.95	47.84	47.65	39.63	---	0/506
EF064853.1	38.69	47.92	49.22	47.95	47.84	47.65	33.82	100.00	---

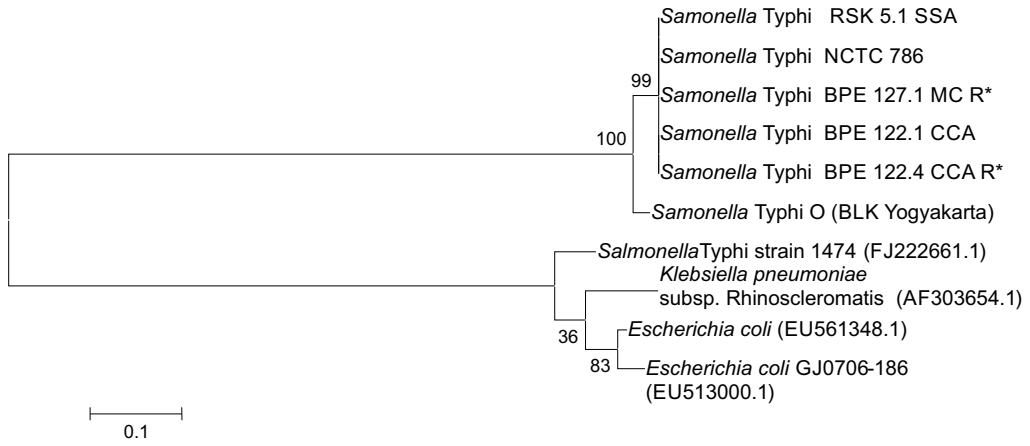

Fig 3 Phylogenetic tree of *Salmonella* strains based on parC sequences analysis. The tree was constructed by neighbor-joining method. Bar, 1 substitution per 10 nucleotides.

Table 4 Nucleotide similarity values (%) and the number of nucleotide differences between *Salmonella* strains based on parC sequence

	*Samonella*Typhi RSK 5.1 SSA	*Samonella*Typhi BPE 122.1 CCA	*Samonella*Typhi NCTC 786	*Samonella*Typhi BPE 122.4 CCA R*	*Samonella*Typhi BPE 127.1 MC R*	*Samonella*Typhi O (BLK Yogyakarta)	*Salmonella*Typhi strain 1474 (FJ222661.1)
*Samonella*Typhi RSK 5.1 SSA	---	0/334	0/331	0/331	3/334	49/330	133/272
*Samonella*Typhi BPE 122.1 CCA	100.00	---	0/332	0/332	3/335	48/330	132/271
*Samonella*Typhi NCTC 786	100.00	100.00	---	0/337	1/337	47/328	132/270
*Samonella*Typhi BPE 122.4 CCA R*	100.00	100.00	100.00	---	0/336	47/328	132/270
*Samonella*Typhi BPE 127.1 MC R*	99.10	99.10	99.70	100.00	---	50/333	135/274
*Samonella*Typhi O (BLK Yogyakarta)	85.15	85.45	85.67	85.67	84.98	---	142/279
*Salmonella*Typhi strain 1474 (FJ222661.1)	51.10	51.29	51.11	51.11	50.73	49.10	---

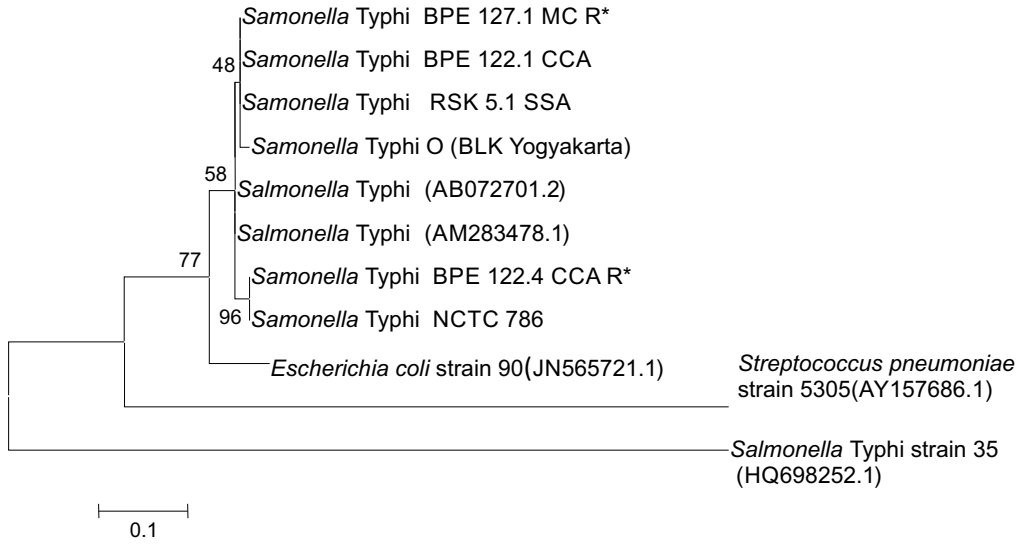

Fig 4 Phylogenetic tree of *Salmonella* strains based on parE sequences analysis. The tree was constructed by neighbor-joining method. Bar, 1 substitution per 10 nucleotides.

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.

Table 5 Nucleotide similarity values (%) and the number of nucleotide differences between *Salmonella* strains based on parE sequence

	*Samonella*Typhi RSK 5.1 SSA	*Samonella*Typhi BPE 127.1 MC R*	*Samonella*Typhi NCTC 786	*Samonella*Typhi BPE 122.4 CCA R*	*Samonella*Typhi BPE 122.1 CCA	*Samonella*Typhi O (BLK Yogyakarta)	*Salmonella* Typhi (AM283478.1)	*Salmonella* Typhi (AB072701.2)	*Salmonella* Typhi strain 35 (HQ698252.1)
*Samonella*Typhi RSK 5.1 SSA	---	0/198	4/199	4/196	0/197	2/197	1/199	1/199	107/193
*Samonella*Typhi BPE 127.1 MC R*	100.00	---	4/198	4/196	0/197	2/197	1/198	1/198	107/192
*Samonella*Typhi NCTC 786	97.99	97.98	---	0/200	4/197	6/200	4/207	4/207	113/203
*Samonella*Typhi BPE 122.4 CCA R*	97.96	97.96	100.00	---	4/196	6/196	3/198	3/198	105/193
*Samonella*Typhi BPE 122.1 CCA	100.00	100.00	97.97	97.96	---	2/196	1/197	1/197	106/191
*Samonella*Typhi O (BLK Yogyakarta)	98.98	98.98	97.00	96.94	98.98	---	3/200	3/200	108/194
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	---

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 phylgenetic 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 similarityamong *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 togetherin 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 factsindicated 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 gryA 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 demostrated that taxonomy based on 16S rRNA typing methods may not be enough for the delineation phylogenetic differences at the spesies level. The 16S

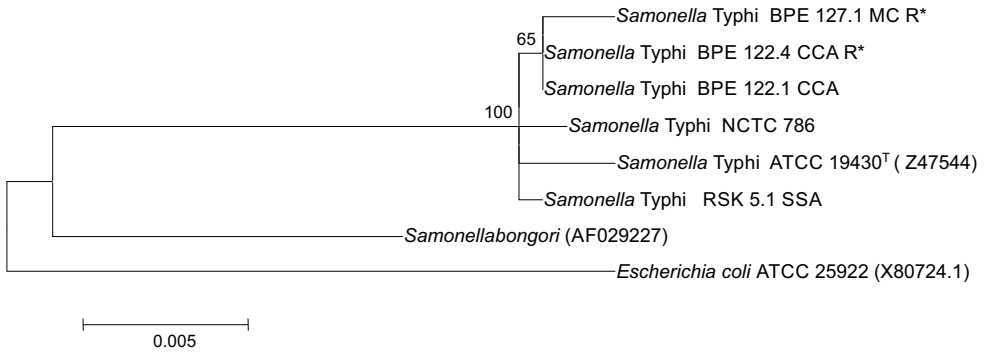

Fig 5 Phylogenetic tree of *Salmonella* strains based on 16S rRNA sequences analysis. The tree was constructed by neighbor-joining method. Bar, 5 substitution per 5000 nucleotides.

Table 6 Nucleotide similarity values (%) and the number of nucleotide differences between *Salmonella* strains based on 16S rRNA

	S. Typhi BPE 122.4 CCA (R*)	*S.* Typhi BPE 127.1 MC (R*)	*S.* Typhi BPE 122.1	*S.* Typhi RSK 5.1 SSA	*S.* Typhi NCTC 786	Z47544	AF029227	X80724.1
S. Typhi BPE 122.4 CCA (R*)	---	3/1383	0/1381	2/1382	3/1381	5/1381	33/1377	45/1374
S. Typhi BPE 127.1 MC (R*)	99.78	---	3/1381	5/1382	6/1381	8/1381	36/1377	48/1374
S. Typhi BPE 122.1	100.00	99.78	---	2/1381	3/1381	5/1381	33/1377	45/1374
S. Typhi RSK 5.1 SSA	99.86	99.64	99.86	---	3/1381	5/1381	33/1377	45/1374
S. Typhi NCTC 786	99.78	99.57	99.78	99.78	---	6/1381	34/1377	46/1374
Z47544	99.64	99.42	99.64	99.64	99.57	---	36/1497	48/1446
AF029227	97.60	97.39	97.60	97.60	97.53	97.60	---	39/1442
X80724.1	96.72	96.51	96.72	96.72	96.65	96.68	97.30	---

rRNA genes couldn't be used as a suitable marker for classification of closely related bacterial spesies (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.

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