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AMINO ACIDS VARIATION AT PARC GENE OF CLINICAL SALMONELLA TYPHI ISOLATES

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Abstract

This study was conducted to find out the best molecular marker for detecting novel Salmonella strains from local area in Indonesia. Two clinical isolates of Salmonella Typhi from different patients in East Nusa Tenggara were examined for its resistance against nalidixic acid. The quinolone resistance-determining regions (QRDR) of the structural genes encoded by parC were amplified by PCR, and the nucleotide sequences of the resistants isolates were then compared to those of the susceptible ones. Analysis of the amino acids sequences of the parC showed that the resistant isolates clustered as a different clade compared to the sensitive strains, which make another clades. There are amino acids differences between two resistant strains being analysed, as well as between the resistant and the sensitive strains.

Key words: *Salmonella* Typhi, clinical isolates, parC, amino acid variation

INTRODUCTION

Salmonella Typhi is one of the pathogenic gram-negative bacteria, member of the family Enterobacteriaceae. This species causes enteric fever in humans. There are over 2,500 serotypes in the genus of *Salmonella*. Species in this genus have been validly classified by two different systems, firstly, the two-species system of *Salmonella enterica* and *Salmonella bongori*. Secondly, the five-species system that differentiate *Salmonella* into five spesies as follows: ***Salmonella arizonae*** (Borman 1957) Kauffmann 1964, *type strain*: ATCC 13314; ***Salmonella choleraesuis*** corrig. (Smith 1894) Weldin 1927, *type strain*: ATCC 13312; ***Salmonella enteritidis*** (Gaertner 1888) Castellani and Chalmers 1919, *type strain*: ATCC 13076; ***Salmonella typhi*** (Schroeter 1886) Warren and Scott 1930, *type strain*: ATCC 19430; and ***Salmonella typhimurium*** (Loeffler 1892) Castellani and Chalmers 1919, *type strain* ATCC 13311 (Skerman *et al.*, 1980). Later, it was found that *Salmonella subterranea* as the new spesies. Therefore in the previous publication, it is reported the three-species system with *Salmonella enterica*, *Salmonella bongori*, and *Salmonella subterranea* (Shelobolina *et al.*, 2004). In this communication we use *Salmonella* Typhi. This reflects that *Salmonella* Typhi is a serotype of *Salmonella enterica* subspecies enterica.

Results of previous studies indicated that *S.* Typhi isolates derived from patients with typhoid fever contained the genetic and biochemical diversity. Some strains of which are also known to be resistant to nalidixic acid (Amarantini *et al.*, 2009; 2010; 2011; 2012). The high diversity among these isolates, indicating the presence of a polymorphism in *S.* Typhi isolates. Furthermore, the use of gyrB gene is proven to increase taxonomic resolution of *Salmonella* members. GyrB gene become a potential marker for detecting the intraspecies diversity of bacteria belonging to *Salmonella* Typhi. Resolution based on the gyr B gene is higher compared

than 16S rRNA gene, characterized by the formation of a clade. Although the use of gyrB gene increase the resolution, but the use of this marker can not differentiate between the sensitive and the resistant strains (Amarantini and Satwika, 2014a). Higher taxonomic resolution was obtained in QRDR region by using four partial marker genes (*ie*. gyrA, gyrB, parC, and parE). Based on the topology of the phylogenetic structure and similarity value, it is known that the highest taxonomic resolution obtained by the use of parC, gyrB, parE and gyrA, respectively (Amarantini and Satwika, 2014b).

Quinolone resistance in *Salmonella* increased since the use of nalidixic acid as an antimicrobial agent. Research shows that the main mechanism of resistance to quinolones is known by detecting mutations through QRDR region. This study aims to detect QRDR region of nalidixic acid resistance of *S*. Typhi isolates using parC gene. This data is useful to determine the mutation through its amino acid changes, perform an assessment of the mutation with resistance to nalidixic acid a strain, and to develop DNA markers for the detection of nalidixic acid resistant strains.

RESEARCH METHOD

Bacterial cultures. Two nalidixic acid-resistant and two nalidixic acid-sensitive isolates were used in this research. We also used the reference strains; i.e. *S*. Typhi NCTC 786 collection from PT Biofarma and *S*. Typhi O from BLK Yogyakarta.

DNA isolation. DNA was isolated by performing the phenol-chloroform-isoamyl alcohol method (Sambrook *et al*., 1989). To isolate chromosomal DNA, 1 ml of overnight culture grown in Brain Heart Infusion were put into a 1.5 ml Eppendorf tube and centrifuged at 5000 rpm, 15 minutes to obtain the cells.

PCR and sequencing of parC gene. Fragment of the parC gene containing the QRDR associated with quinolone resistance were amplified by PCR. The PCR was done using DreamTaq™ Green PCR Master Mix in total reaction volume of 50µl containing 25 µl of 2x DreamTaq™ Green PCR Master Mix, 1 µl of 1 µM primer stocks, and 1 µl of template DNA. Spesific primer for parC gene (F, 5' ATgAgCgATATggCAgAgCg 3'; R, 5' TgACCgAgTTCgCTTAACAg 3') was used to amplify a 412 bp of DNA fragment (Ling *et al*. 2003). The PCR reaction mixtures were amplified for 35 cycles with pre-denaturation at 95ºC for 5 minute, denaturation at 94ºC for 1 minute, annealing at 52ºC for 1 minute, and extention at 72ºC for 1 minutes, with a final extension at 72ºC for 5 minutes. 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.

Sequencing of parC gene and amino acid 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). Multiple alignment of nucleotide or amino acid sequences was performed using ClustalX 2.1 (Larkin *et al*., 2007), reconstructed with MEGA5 (Tamura *et al*., 2011) and edited with GeneDoc.2.7 (www.psc.edu/biomed/genedoc).

RESULT AND DISCUSSION

The goal of the present study was to identify and characterize parC gene associated with quinolone resistance in Na^R *Salmonella* Typhi isolates. Specific primer pair (F, 5' ATgAgCgATATggCAgAgCg 3'; R, 5' TgACCgAgTTCgCTTAACAg 3') were used to amplify Na^R *Salmonella* Typhi (BPE 122.4 CCA and BPE 127.1 MC) genomic DNA sequences containing the QRDR region of the parC gene. The purified amplification products were sequenced and their nucleotide sequences were compared to that of the parC QRDR from genebank data. As expected, an amplification product of 412 bp was obtained (Figure 1). The DNA sequences shared 100% identity with the parC protein of *Salmonella enterica* subsp. enterica serovar Typhi (ABU89281.1). Based on the high percentage of similarity with the parC protein, it seems highly probable that the amplified DNA fragment of BPE 122.4 CCA and BPE 127.1 MC isolates represents the parC QRDR of *Salmonella* Typhi. These results are consistent with the phylogenetic analysis (Amarantini and Satwika, 2014b).

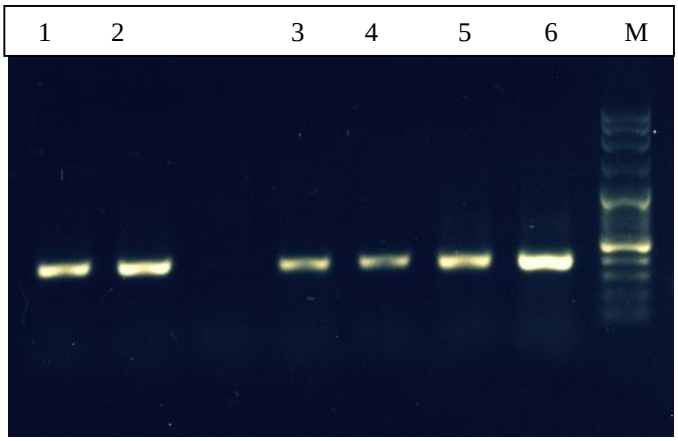

Figure 1. Amplification product (412 bp) of the parC gene of QRDR in Na^R *Salmonella* Typhi isolates. Lane 1-2: *Salmonella* Typhi NCTC 786 and *Salmonella* Typhi O (BLK Yogyakarta). Lane 3-4: amplicon of Na^S *Salmonella* Typhi isolates. Lane 5-6: amplicon of Na^R *Salmonella* Typhi isolates (BPE 122.4 CCA and BPE 127.1 MC).

Figure 2 showed the analysis result of translated product of parC indicating the amino acid variation of resistant isolates (Na^R *Salmonella* Typhi isolates). As shown in Figure 2, additional asparagine (N) was found at amino side, whereas additional histidine (H), phenylalanine (F), isoleucine (I) and valine (V) were observed at carboxyl side in Na^R *Salmonella* Typhi isolates. Interestingly, no signal peptide is detected (http://www.cbs.dtu.dk/services/SignalP/), indicating that this protein is not excreted from the cells.

Aparagine is classified in the group of amino acids with polar uncharged side chains, which means it will be active at neutral pH. Interestingly, phenylalanine, isoleucine, leucine, and valine classified in amino acids with hydrophobic side chains (Berg *et al*., 2011). Report already arise the importance of those amino acids in the secondary structure stability of a periplasmic binding protein in *Escherichia coli* (Sack *et al*., 1989). Phenylalanine was reported to play important role in binding the peptide and protein and in aligning the catalytic site of the complex formed (Lin *et al*., 1972). If this type of amino acids found in the resistant group and the signal peptide analysis indicating that the protein product of parC gene is not excreted, it is suspected the possibility of this variation occurs as a form of self-protection. To determine

whether these amino acids could be involved in their resistance to quinolone, further research is needed to elucidate the function of these amino acid.

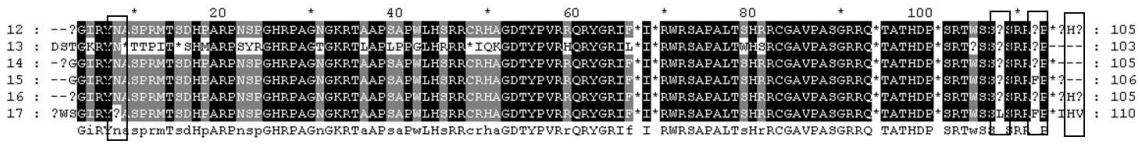

Figure 2. Alignment of the amino acid sequence of the parC QRDR of Na^R *Salmonella* Typhi isolates (no. 16-17) with those Na^S *Salmonella* Typhi isolates (no. 14-15), *Salmonella* Typhi NCTC 786 (no. 12) and *Salmonella* Typhi O (BLK Yogyakarta) as positive control isolates. Boxes indicating additional amino acids detected from the resistant strains.

CONCLUSION AND SUGGESTION

There are amino acids differences between two resistant strains being analysed, as well as between the resistant and the sensitive strains. Asparagine is known to exist at the at $–NH_3$ side, whereas phenylalanine, isoleucine, leucine, and valine is known to exist at the –COOH side. So far, the important role of this amino acid is not known. Therefore, it deserve further research to clarify the findings in this study.

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