Phylogenetic relationship of Gram Negative Bacteria of Enterobacteriaceae Family in the Positive Widal Blood Cultures based on 16S rRNA Gene Sequences

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Abstract
The purpose of this study was to analyze the phylogenetic relationship of Gram negative bacteria (3 strains of Salmonella typhi, 1 strain of Escherichia coli, 1 strain of Serratia marcescens, and 3 strains of Enterobacter cloacae) of Enterobacteriaceae family in positive Widal blood cultures based on 16S rRNA gene sequences. The results respectively showed that each two 16S rRNA gene clones of Serratia marcescens KD 08.4 had a close relationship with 16S rRNA gene of Serrratia marcescens ATCC 13880 (similarity: 99.53-99.8%), Eschericia coli BA 30.1 with Eschericia coli ATCC 11775T (similarity: 99.38-99.67%), Salmonella typhi BA 07.4, Salmonella typhi KD 30.4, and Salmonella typhi SA 02.2 with Salmonella typhi ATCC 19430T (similarity: 99.4-100%) as well as the isolates of Enterobacter cloacae SA 02.1, Enterobacter cloacae BA 45.4.1, one 16S rRNA gene clone of Enterobacter cloacae TG 03.5 with Enterobacter cloacae ATCC 23373 (similarity: 99.0-99.87%).

Keywords: Widal, Enterobacteriaceae, 16S rRNA genes

Introduction
The typhoid fever incidence rate in Indonesia had reached 358-810/100,000 population/year with the mortality rate of 1-5% of patients (Anonymous, 2007). In Semarang, typhoid fever had been in the third rank of 10 major diseases after Dengue Fever and Diarrhea and gastroenteritis (Anonymous, 2008). Typhoid fever was a serious systemic infectious disease that was possibly accompanied by a variety of diseases such as dengue fever and malaria (Gasem et al., 2002).

The clinical features of typhoid fever were unspecific that the gold standard diagnosis could not only depend on the clinical symptoms but it should also be supported by the laboratory diagnosis (Khoharo et al., 2010; Ley et al., 2010; Fadeel et al., 2011). The gold standard diagnosis of typhoid fever was by the finding of Salmonella typhi (S. typhi) in blood or bone marrow cultures (Khoharo et al., 2010; Ley et al., 2010). However, the facilities for culturing of bacteria were not always available, it was expensive, time consuming (seven days) and the result was frequently negative since the patients had consumed antibiotics. Widal test was a widely used laboratory test in Indonesia supporting the typhoid fever diagnosis as it was cheap, easy, fast, and simple. The sensitivity, specificity,
and predictive values of Widal test were various, due to the presence of anti-O and anti-H antibodies in patients infected by *Salmonella* sp., species of *Enterobacteriaceae* family member other than *Salmonella* sp. and malaria (Novianti, 2006; Beig et al., 2010). Darmawati et al., 2012, stated that there was bacterial species diversity of *Enterobacteriaceae* family members of such as *S. typhi*, *Serratia marcescens*, *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae* in positive Widal blood cultures from Semarang. However, the phylogenetic relationship between species was unknown. Thus, the purpose of this study was to determine the phylogenetic relationship of Gram-negative bacillus bacteria of *Enterobacteriaceae* family members based on 16S rRNA gene sequences.

**Materials And Methods**

**Bacterial Strains**

There was a total of 8 isolates (3 isolates of *Salmonella typhi*, 1 isolate of *Escherichia coli*, 1 isolates of *Serratia marcescens*, and 3 isolates of *Enterobacter cloacae*) isolated from positive Widal blood samples of in and outpatients from Semarang (Tugurejo hospital, City Hospital of Semarang, Sultan Agung Islamic Hospital, Community Health Center of Bangetayu, and Community Health Center of Kedungmundu). The bacterial Identification used API 20E and API 50CHB/E media (Darmawati, et al., 2012).

**DNA bacterial Extraction, PCR amplification, cloning, and DNA plasmid extraction with insertion and sequencing**

DNA was extracted from eight bacterial strains used DNeasy Blood & Tissue Kits (Qiagen, K69504). The 16S rRNA gene amplification used Applied Biosystems GeneAmp PCR System 2400, 0.25μl Takara Ex Taq, 5μl 10X Ex Taq buffer, 4 μl dNTP Mixture (2.5 mM each), 2μl DNA template, 0.5μl primer 8F (1.0μM final conc.), and 0.5μl primer 4192R (1.0μM final conc.), 37.75μl sterile deionized water, for a total volume of 50μl. The thermal cyling was as follows: denaturation at 95° C for 30 sec, annealing at 55° C for 30 sec, extension at 72° C for 1.5 min, and final extension 72° C for 10 min for the total of 30 cycles. PCR products (1500bp) were visualilized through electrophoresis at 1% agarose gel with ethidium bromide added directly to the gel.

The amplified DNA bands were purified from agarose using glass powder method (Volgstein and Gillespie, 1979), ligated to T-Vector pMD20 (Takara Biotechnology), and transformed to *E.coli* DH5α. The plasmid DNA containing inserts was isolated, respectively amplified using primer M13 reverse, U515F, and M13-40 (Table 1). The amplified DNA were sequenced using primer M13 reverse, U515F, and M13-40. The DNA Sequencing was conducted with sequencer device of ABI Prism™ 310 Genetic Analyzer. The sequenced data were in the form of electrophenogram files and base DNA arrangement.

Tabel 1. Primer for 16S rRNA gene amplification dan sequencing

| Primer   | Sequences                |
|----------|--------------------------|
| 8F       | 5'-AGA GTT TGA TCC TGG CTC AG-3' |
| 1492 R   | 5'-AAG TCG TAA CAA GGT AAC C-3' |
| M13-RV   | 5'-CAG GAA ACA GCT ATG AC-3' |
| U515F    | 5'-GTG CCA GCC GCC GCG GTA A-3' |
| M13-40   | 5'- GTT TTC CCA GTC ACG AC-3' |

**Analysis and alignment of 16S rRNA gene sequences**

The 16S rRNA sequence were analyzed and compared to the Gene Bank nucleotide database using Basic Local Alignment Search Tool (BLAST). The 16S rRNA Sequences of 8 bacterial strains were aligned using CLUSTAL X program.

**Phylogenetic tree Construction**

Phylogenetic tree was prepared using PHYLIP program, matrix similarity, and nucleotide difference of 16S rRNA between clones and strains analyzed with PHYDIT program.
Results And Discussion

The results of 16S rRNA gene amplification of 8 isolates of Enterobacteriaceae family members were shown in Figure 1 while the results of phylogenetic relationship analysis based on 16S rRNA gene sequences were shown in Figure 2. There was a total of 15 sequences with each isolate consisted of two 16S rRNA gene sequences derived from two clones of 16S rRNA gene sequences except TG 03.5 isolate and 5 sequences derived from 16S rRNA sequences of Gram-negative bacillus bacteria of Enterobacteriaceae family (Gene Bank, NCBI) consisting of S. typhi strain types of ATCC 19430T (accession no. Z47544), E. coli 11775T (X80725.1), Ent. cloacae ATCC 23373 (HQ651841.1), Citrobacter freundii ATCC 8090 (AJ233408.1), Ser. marcescens ATCC 13880 (AB594756.1). Two strains as out group used Vibrio cholerae ATCC 14547 (NR_044050.1) from Vibrionaceae family (negative catalase and positive oxidase) and Pseudomonas aeruginosa ATCC 23993 (FJ652615.1) from Pseudomonadaceae family (positive catalase and oxidase).

After the 16S rRNA gene sequences was aligned with Clustal-X program, the phylogenetic trees were arranged using PHYLIP program

The phylogenetic relationship analysis of 15 16S rRNA gene clones of 8 isolates (Figure 2) was divided into five clades. The first clade consisted of 6 16S rRNA gene clones derived from 3 isolates (SA 02.2, KD 30.4, and BA 07.4) and 2 reference strains: S. typhi ATCC 19430T and Ent. cloacae ATCC 23373. The similarity value of those six clones with S. typhi ATCC 19430T was 99.4 to 100% with the difference of 0-9 nucleotide, shown in Table 2.

Table 2. Matrix similarity and nucleotide difference of 16S rRNA gene sequences of Gram negative bacillus bacteria of Enterobacteriaceae family members on the first clade with reference strains of S. typhi ATCC 19430T

| Isolate Code | SA 022C3 | SA 022C1 | BA 074C1 | KD 304C1 | KD 304C3 | BA 074C3 | S. typhi ATCC 19430T | Ent. cloacae ATCC 23373 |
|--------------|----------|----------|----------|----------|----------|----------|-------------------|------------------------|
| SA022C3      | ---      | 4/1502   | 2/1503   | 2/1503   | 9/1500   | 5/1500   | 9/1503            | 27/1498                |
| SA022C1      | 99.73    | ---      | 2/1502   | 2/1502   | 9/1499   | 5/1499   | 9/1502            | 27/1497                |
| BA074C1      | 99.87    | 99.87    | ---      | 0/1503   | 7/1500   | 3/1500   | 7/1503            | 25/1498                |
| KD304C1      | 99.87    | 99.87    | 100      | ---      | 7/1500   | 3/1500   | 7/1503            | 25/1498                |
| KD304C3      | 99.4     | 99.4     | 99.53    | 99.53    | ---      | 6/1499   | 9/1500            | 28/1495                |
| BA074C3      | 99.67    | 99.67    | 99.8     | 99.8     | 99.6     | ---      | 3/1500            | 22/1495                |
| S. typhi ATCC 19430T | 99.4 | 99.4 | 99.53 | 99.53 | 99.4 | 99.8 | --- | 27/1504 |
| Ent. cloacae ATCC 23373 | 98.2 | 98.2 | 98.33 | 98.33 | 98.13 | 98.53 | 98.2 | --- |
Based on the nucleotide similarity value, it could be concluded that isolate SA 02.2, KD 30.4, and BA 07.4 were identified as *S. typhi* members. Two different 16S rRNA clones, derived from one isolate also showed the presence of nucleotide differences of 3-7 (Similarity 99.53 to 99.80%).

The second clade consisted of two 16S rRNA gene clones derived from one isolate BA 30.1, with reference strain of *E. coli* ATCC 19430\(^7\) in the similarity value between 99.38 to 99.67% with nucleotide differences of 5-9 (Table 3). The isolate BA 30.1, based on its close relationship with the reference strain was identified as *E. coli* members.

The third clade consisted of five 16S rRNA gene clones derived from 3 isolates (BA 45.4.1, SA 02.1 and TG 03.5). The relationship between three bacterial isolates from Bangetayu Community health center, Tugurejo hospital and Sultan Agung Islamic Hospital showed the similarity value of 99.0 - 99.87% with nucleotide difference of 2-15 (Table 4), which had the closest kinship relationship with *Ent. cloacae* ATCC 23373.

| Isolate Code | BA 301C1 | BA 301C3 | E. coli ATCC11775T |
|--------------|---------|---------|--------------------|
| BA 301C1     | ---     | 5/1503  | 8/1447             |
| BA 301C3     | 99.67   | ---     | 9/1447             |
| *E. coli* ATCC11775T | 99.45   | 99.38   | ---                |
The number of 16S rRNA gene copies in bacteria was various (1-15) in each genome. Each copy had the size approximately 1500 bp. Marchandin et al. (2003) reported that the 16S rRNA gene sequences in each copy of every organism were identical. The nucleotide difference of 16S rRNA gene copies was called micro-heterogeneity. The 16S rRNA gene sequences in 2 different 16S rRNA gene clones from the same isolates showed the presence of nucleotide difference with the similarity value of 99-100%. The results of this study was similar with the findings of Marchandin et al. (2003), that 4 16S rRNA gene copies in one bacterial strain of Veillonella sp. ADV 360.1 showed two identical gene copies (similarity 100 %) and two various gene copies (similarity 98.5 to 99.8%).

The fourth clade consisted of one reference strain of Citrobacter freundii ATCC 8090. The fifth clade consisted of 2 16S rRNA gene clones derived from isolate KD 08.4 (similarity 99.53-99.8 %) and one reference strains of Ser. marcescens ATCC 13880 with nucleotide difference number of 3-7 nucleotides, were shown in Table 5.

Based on the nucleotide similarity value, it could be concluded that isolate KD 08.4 was identified as the isolate of Ser. marcescens KD 08.4 included as Ser. marcescens members.

The constructed phylogenetic trees based on Neighbor-Joining algorithm (Saitou and Nei, 1987) showed the relationship between 8 isolates of Gram-negative bacillus bacteria obtained from patients’ positive Widal blood samples, included to Enterobacteriaceae family isolates which each consisted of 2 16S rRNA gene clones, except isolate TG 03.5, with 5 reference strains (Ser. marcescens ATCC 13880, Cit. freundii ATCC 8090, Ent. cloacae ATCC 23373, E. coli ATCC 11775T, S. typhi ATCC 19430) based on 16S rRNA gene sequences. The data showed that the isolates found in positive Widal blood were 8090. The fifth clade consisted of 2 16S rRNA gene clones derived from isolate KD 08.4 (similarity 99.53-99.8 %) and one reference strains of Ser. marcescens ATCC 13880 with nucleotide difference number of 3-7 nucleotides, were shown in Table 5.

Table 4. Matrix similarity and nucleotide difference of of 16S rRNA gene sequences of Gram negative bacillus bacteria of Enterobacteriaceae family members on the third clade and reference strain of Ent. cloacae ATCC 23373

| Isolate Code | BA 4541C3 | TG 035C1 | SA 021C2 | BA 4541C2 | SA 021C3 | Ent. cloacae ATCC 23373 |
|--------------|-----------|----------|----------|-----------|----------|-------------------------|
| BA4541C3     | ---       | 2/1500   | 7/1500   | 6/1499    | 12/1499  | 32/1495                |
| TG035C1      | 99.87     | ---      | 5/1502   | 4/1501    | 10/1502  | 30/1498                |
| SA021C2      | 99.6      | 99.67    | ---      | 9/1501    | 15/1501  | 31/1497                |
| BA4541C2     | 99.6      | 99.73    | 99.4     | ---       | 12/1500  | 32/1496                |
| SA021C3      | 99.2      | 99.33    | 99       | 99.2      | ---       | 23/1497                |
| Ent. cloacae ATCC23373 | 97.86 | 98 | 97.93 | 97.86 | 98.46 | --- |

Table 5. Matrix similarity and nucleotide difference of 16S rRNA gene sequences of Gram negative bacillus bacteria of Enterobacteriaceae family members on the fifth clade and reference strain of Ser. marcescens ATCC13880

| Isolate Code | KD 084C3 | KD 084C1 | Ser. marcescens ATCC13880 |
|--------------|----------|----------|--------------------------|
| KD084C3      | ---      | 3/1502   | 7/1488                    |
| KD084C1      | 99.8     | ---      | 6/1489                    |
| Ser. marcescens ATCC13880 | 99.53 | 99.6 | --- |
Besides using numerical systematic based on phenotypic characters and chemical systematic, the bacterial identification could also conducted by using the molecular character systematic based on the nucleic acids, such as based on 16S rRNA genes. 16S rRNA genes were conserved genes which were present in all bacteria, that could be used to classify bacteria based on the kinship relationship. The genomic analysis was better than the protein analysis since it did not rely on certain genomic expression which encoded proteins and might result in phenotypic variation (Priest and Austin, 1995; Vandamme et al., 1996; Giammanco et al., 1999).

The results of phylogenic analysis based on 16S rRNA gene sequences showed that the isolates presented in positive Widal blood were *Ser. marcescens* KD 08.4 which had close relationship with *Ser. marcescens* ATCC 13880, *E. coli* BA 30.1 with *E. coli* ATCC 11775T, *S. typhi* BA 07.4, *S. typhi* KD 30.4, and *S. typhi* SA 02.2 with *S. typhi* ATCC 19430T, and the isolates of *Ent. cloacae* SA 02.1, *Ent. cloacae* BA 45.4.1, *Ent. cloacae* TG 03.5 with *Ent. cloacae* ATCC 23373. The similarity value of 8 isolates (15 16S rRNA gene clones) was between 97.86 to 100%. The Research conducted by Darmawati et al. (2013), showed that the classification based on biochemical characters was congruent with the results of classification based on total protein. This was also congruent with the classification based on 16S rRNA gene sequences. It might happen as the biochemical characteristics was the reflection of enzyme activities. The enzyme was functionally active protein as a result of translated expressed gene in a genome. Thus, the classification combining between the classification based on phenotypic, chemical, and molecular might result in accurate classification.

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