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Identification of *Staphylococcus* sp. strains isolated from Positive Widal Blood Based on 16s rRNA gene sequences

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Abstract- *The purpose of this study is to identify 8 strains of Staphylococcus genus members isolated from positive Widal blood (4 strains of Staphylococcus saprophyticus, 1 strain of Staphylococcus warneri, 2 strains of Staphylococcus hominis, 1 strain of Staphylococcus xylosus and 1 strain of Staphylococcus capitis) based on 16S rRNA gene sequences. The methods used in this study are conducting PCR of 16S rRNA gene, cloning genes using T-Vector pMD20 which is transformed to Escherichia coli DH5α, sequencing. The results show that four strains (BA 47.4, BA 19.2, KD 29.5 and TG 09.1) are identified as Stap. saprophyticus strains of Stap. saprophyticus members of ATCC 15305^T (99.01-100% similarity). The strain of TG 01.3 is identified as Stap. warneri strain of TG 01.3 of Stap. warneri members of ATCC 27836^T (99.74-99.93% similarity), 2 strains (KT 29.2 and KD 35.1) are identified as of Stap. hominis members of DSM 20328^T (99.4-99.67% similarity). The strain of KT 30.5 is the only member of the fifth clade with 78.4-78.9% similarity if compared with members of the fourth clade.*

Keywords—*Staphylococcus, Widal, 16S rRNA gene*

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I. INTRODUCTION

Staphylococcus genus which consists of 42 species and subspecies of Gram-positive coccus bacteria, positive catalase, negative oxidase, facultative anaerobes, including *Micrococcaceae* family (Ghebremedhin et al., 2008) may cause nosocomial infections ranging from mild skin infections to bacteremia (Li et al . 2009). *Staphylococcus* which is spread in various environments such as soil, seawater, fresh water, dust and air, has natural population found on skin and mucous membranes of warm-blooded animals including humans (Sudagi et al. 2005; Wieser, & Busse, 2000). Initially, only *Staphylococcus aureus* with positive coagulase is pathogenic. However, in the last two decades, *Staphylococcus* with negative coagulase appears as pathogen, especially in patients with invasive measures and immunocompromised (Wieser & Busse 2000). Infection in bloodstream is a quite dangerous condition that may cause death in quite high rate (Wellinghausen, et al., 2009). Blood cultures are standards for microbiological infection diagnosis in bloodstream. Blood cultures are usually less sensitive since patients have typically been taking antibiotics. In addition, conventional culture and identification take longer time (2-7 days) (Darmawati et al., 2014; Woo, et al., 2001).

In contrast, DNA-based procedure is faster and more reliable diagnoses. Bacterial identification may be conducted by PCR and 16S rRNA gene sequencing with more accurate results (Ghebremedhin et al. 2008). Thus, the purpose of this study is to identify the strains of *Staphylococcus* genus members based on 16S rRNA gene sequences.

II. MATERIAL AND METHOD

Strains of Bacteria

The bacterial strains used are 8 isolates (4 isolates of *Staphylococcus saprophyticus*, 1 isolate of *Staphylococcus warneri*, 2 isolates of *Staphylococcus hominis*, 1 isolate of *Staphylococcus xylosus* and 1 isolate of *Staphylococcus capitis*), which are isolated from positive Widal blood of in and outpatients from Semarang (PHC of Kedungmundu, PHC of Bangetayu, Tugurejo hospital, and Semarang Hospital). Bacterial identification is conducted using *Rapid Test Kit* API Stap (Darmawati et al. 2015).

DNA bacterial extraction, PCR amplification, cloning, DNA plasmid extraction through insertion and sequencing

DNA is extracted from eight strains of bacteria using *DNeasy Blood & Tissue Kits* (Qiagen, K69504). The amplification of 16S rRNA gene is conducted using *Applied Biosystems GeneAmp PCR System of 2400*, 0.25 l of Takara Ex Taq, 5 l 10X of *Ex Taq* buffer, 4 mL of dNTP Mixture (2.5 mM each), 2 l of DNA template, 0,5 l of primary 8F (1.0 M of final conc.), 0.5 l of primary 4192R (1.0 M of final conc.), 37.75 l of sterile deionized water, with a total volume of 50 l. The thermal cycles are as follows: denaturation at 95 ^{0}C for 30 sec, annealing at 55 ^{0}C for 30 sec, extension at 72 ^{0}C for 1.5 min, and final extension of 72 ^{0}C for 10 min for a total for 30 cycles. PCR products (1500bp) are visualized through electrophoresis on 1% of agarose gel with ethidium bromide which is directly added to the gel (Darmawati et al. 2014).

DNA resulted from amplification of agarose purification using glass powder method (Vogelstein & Gillespiet 1979) is legated to T-Vector pMD20 (Takara Biotechnology) and is transformed to *E. coli* DH5α competent cell. The inserted containing clones isolated by DNA plasmids, respectively amplified using reversed primary M13 are U515F and M13-40 (Table 1). The amplified results which are sequenced using reversed primary M13 are U515F and M13-40. DNA sequencing is conducted using sequencer device of *ABI Prism™ 310 Genetic Analizer*. The results of sequencing are *electrophenogram* file and the sequence of base DNA.

Table 1. Primaries for 16S rRNA gene amplifications and sequencing (Darmawati et al. 2014)

Primary	Sequence
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 GCA 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 sequences are analyzed and Compared to GenBank nucleotide database using Basic Local Alignment Search Tool (BLAST). 16S rRNA sequences from 8 bacterial strains are aligned using CLUSTAL X program.

Phylogenetic tree Constructions

Phylogenetic tree is arranged using PHYLIP program, matrix similarity, and differences between 16S rRNA nucleotide inter-clones and inter-strains analyzed using PHYDIT program.

III. RESULT AND DISCUSSION

Analysis on phylogenetic relationships of 15 16S rRNA gene clones from 8 isolates based on 16S rRNA gene sequences, which each isolate consists of 2 16S rRNA gene clones, except the isolate of KT 30.5 is shown in Figure 1. 8 sequences derived from Staphylococcus genus species members (Gene Bank, NCBI), consisting of *Stap. hominis* of DSM 20328^T(X66101.1), *Stap. warneri* of ATCC 27836^T (L37603.1), *Stap. aureus* of ATCC 12600^T(D83357.1), *Stap. aureus* of ATCC 43300 (AM980864.1), *Stap. epidermidis* of ATCC 14990^T (L37605.1), *Stap. capitis* of ATCC 27840 (L37599.1), *Stap. xylosus* of ATCC 29971^T (D83374.1), and *Stap. saprophyticus* of ATCC 15305^T (D833371) are used as comparators. One strain used as outside group is *Streptococcus agalactiae* of ATCC 1381 (AB002479.1), which is included in *Streptococcaceae* family members.

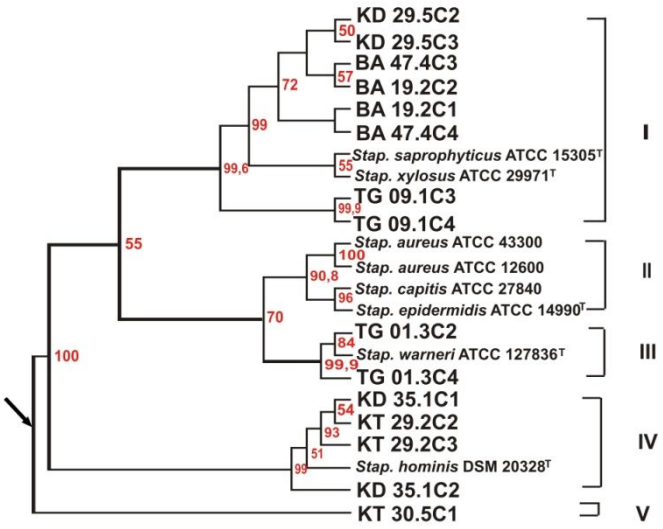

Figure 1. The phylogenetic tree constructed by *algorithm Neighbor Joining* (Saitou and Nei, 1987) shows kinship relation between 8 bacterial isolates (15 clones) of *Staphylococcus* genus of *Micrococcaceae* family members from Semarang, with 8 bacterial strains of *Staphylococcus* genus as references based on 16S rRNA gene sequences. Root locating Determination is conducted using *outgroup* strains of *Streptococcus agalactiae* of ATCC 13813

Based on the results of analysis on phylogenetic relationship of 15 16S rRNA gene clones, *Stapylococcus* genus members are divided into four *clades*. **First *clade***, consists of 8 clones derived from four isolates of BA 47.4, BA 19.2, KD 29.5 and TG 09.1 which are respectively taken from PHC of Bangetayu, PHC of Kedungmundu, and Tugurejo Hospital while *Stap. xylosus* of ATCC 29971^{T}dan *Stap. saprophyticus* of ATCC 1530T as reference strains, at similarity value of 99.01-100% with the difference of 0-18 nucleotides shown in Table 2. Based on nucleotide similarity value to reference strains, those four isolates are identified as *Stap. Saprophyticus* isolates of *Saprophyticus* members of ATCC 15305^T.

Table 2. Matrix of 16S rRNA nucleotide gene sequence similarities and differences of Gram-positive coccus bacteria of *Staphylococcus* genus of *Micrococcaceae* family on the first *clade* and reference strains of *Stap. xylosus* of ATCC 29971^T and *Stap. saprophyticus* of ATCC 15305^T

Isolate Code	BA474C3	BA192C2	KD295C2	KD295C3	BA474C4	BA192C1	*Stap. saprophyticus* ATCC15305^T	*Stap. xylosus* ATCC 29971^T	TG091C3	TG091C4
BA474C3	---	4/1511	4/1510	4/1510	3/1511	3/1511	5/1511	7/1474	18/1511	16/1510
BA192C2	99.74	---	2/1513	2/1513	3/1514	3/1514	5/1514	6/1477	16/1514	16/1513
KD295C2	99.74	99.87	---	0/1513	1/1513	1/1513	3/1513	4/1476	14/1513	14/1513
KD295C3	99.74	99.87	100	---	1/1513	1/1513	3/1513	4/1476	14/1513	14/1513
BA474C4	99.8	99.8	99.93	99.93	---	0/1514	2/1514	4/1477	15/1514	13/1513
BA192C1	99.8	99.8	99.93	99.93	100	---	2/1514	4/1477	15/1514	13/1513
Stap. saprophyticus ATCC15305T	99.67	99.67	99.8	99.8	99.87	99.87	---	3/1477	15/1514	13/1513
Stap. saprophyticus ATCC15305T	99.53	99.59	99.73	99.73	99.73	99.73	99.8	---	14/1477	13/1476
TG091C3	98.81	98.94	99.07	99.07	99.01	99.01	99.01	99.05	---	2/1513
TG091C4	98.94	98.94	99.07	99.07	99.14	99.14	99.14	99.12	99.87	---

The third *clade* which consists of two clones is derived from one isolate of TG 01.3, with *Stap. warneri* of ATCC 27836^T as reference strain, at similarity value of 99.74-99.93% with 1-4 nucleotide differences shown in Table 3. Based on nucleotide similarity value, it can be concluded that the isolate of TG 01.3 is identified as *Stap. warneri* isolate of TG 01.3 of *Stap. warneri* members of ATCC 27836^T.

Table 3. Matrix of 16S rRNA nucleotide gene sequence similarities and differences of Gram-positive coccus bacteria of *Staphylococcus* genus of *Micrococcaceae* family on the third clade and reference strains of *Stap. warneri* of ATCC 27836^T

Isolate Code	*Stap. warneri* ATCC 27836T	TG013C2	TG013C4
Stap. warneri ATCC 27836^T	---	1/1469	3/1468
TG013C2	99.93	---	4/1513
TG013C4	99.8	99.74	---

The fourth *clade* which consists of 4 clones is derived from two isolates (KT 29.2 and KD 35.1) as well as the reference strain of *Stap. hominis* of DSM 20328^T, at similarity value of 99.4-99.67%, with nucleotide number differences of 5-9, as shown in Table 4. Based on nucleotide number similarity and difference value, both isolates are identified as *Stap. hominis* member of DSM 20328^T. The isolate of KT 30.5 is the only member of fifth *clade*. When compared with the fourth *clade* members, the nucleotide similarity value is 78.4-78.9%, supported with large enough nucleotide number differences of 311-317 nucleotides.

Table 4. Matrix of 16S rRNA nucleotide gene sequence similarities and differences of Gram-positive coccus bacteria of *Staphylococcus* genus of *Micrococcaceae* family on the fourth and fifth *clade* with reference strains of *Stap. hominis* of DSM 20328^T

Isolate Code	KT292C3	KT292C2	KD351C1	*Stap. hominis* DSM 20328^T	KD351C2	KT305C01
KT292C3	---	6/1510	6/1512	5/1508	7/1512	313/1474
KT292C2	99.6	---	8/1510	7/1506	9/1510	317/1472
KD351C1	99.6	99.47	---	7/1508	9/1512	315/1474
Stap. hominis DSM 20328T	99.67	99.54	99.54	---	7/1508	313/1471
KD351C2	99.54	99.4	99.4	99.54	---	311/1474
KT305C01	78.77	78.46	78.63	78.72	78.9	---

The phylogenetic tree constructed based on Neighbor-joining algorithm (Saitou and Nei 1987) shows kinship relation between 8 isolates of Gram-positive coccus bacteria of *Staphylococcus* genus of *Micrococcaceae* family members on positive Widal blood of the patients. Each isolate consists of two 16S rRNA gene clones, except KT 30.5 isolate, with 8 reference strains (*Stap. hominis* of ATCC 27844, *Stap. warneri* of ATCC 27836, *Stap. aureus* of

ATCC 14458, *Stap. aureus* of ATCC 43300, *Stap. epidermidis* of ATCC 14990, *Stap. capitis* of ATCC 2784, *Stap. xylosus* of ATCC 299971, *Stap. saprophyticus* of ATCC 1530) based on 16S rRNA gene sequences. The construction results show that Gram-positive coccus bacterial isolates which are contained in positive Widal blood isolates are *Stap. hominis* of KD 35.1 and *Stap. hominis* of KT 29.2, which are respectively derived from PHC of Bangetayu, PHC of Kedungmundu, and Semarang hospital. One isolate of *Stap. warneri* of TG 01.3 is derived from Tugurejo hospital while 4 isolates of *Stap. saprophyticus* of TG 09.1, 47.4 BA, BA KD 19.2 and 29.5 are respectively from Tugurejo Hospital, 2 isolates from PHC of Bangetayu and 1 isolate from PHC of Kedungmundu (Takahashi et al., 1999). Based on 16S rRNA sequence analysis on 38 taxa of *Staphylococcus* genus members suggests that the isolates of *Stap. hominis* and *Stap. warneri* are included in *stap. epidermidis* species members, while the isolates of *Stap. saprophyticus* are included in *Stap. saprophyticus* species members.

The 16S rRNA genes found in all bacterial species are determined *conserved* since mutations are very slow at a constant speed. Thus, it may be used as the *molecular clock* and to construct the phylogenetic tree (Woo, et al., 2001). 16S rRNA gene sequences may be used to identify bacteria with confusing biochemistry properties for identification of bacteria that cannot be cultured (Woo, et al., 2001).

IV. CONLUSIONS

The 16S rRNA gene sequence can be used for identification of *Staphylococcus* sp. strains isolated from positive widal blood.

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