Molecular Confirmation and Detection of Genes Encoding for Enterotoxin Production in *Staphylococcus aureus* Food Isolates

Ratih Dewanti-Hariyadi¹,², Fidyatun Khoiriyah¹, and Sri Hendrastuti Hidayat³

¹Department of Food Science and Technology
²Southeast Asia Food Agriculture Science & Technology (SEAFAST) Center
³Department of Plant Pathology

Bogor Agricultural University, Bogor, Indonesia
Introduction

*Staphylococcus aureus*
- a non sporeformer, Gram positive bacterium; cocci, single or in group, grape-like in shape
- diameter 0.5-1.5 µm, aerobic or facultatively anaerobic, non motile,
- belongs to Staphylococcaceae family, coagulase, catalase positive and capable of mannitol fermentation
- commonly found in human skin, nose, throat
- have been isolated from various foods and clinical isolates
- local isolates were obtained from steamed coconut rice and shredded chicken (Dewanti-Hariyadi et al. 2011); biochemically confirmed but not known to form toxins

Introduction

- S. aureus causes foodborne intoxication due to S. aureus enterotoxins (SE): SEA, SEB, SEC (SEC1,SEC2,SEC3), SED, SEE, SEG, SEH, SEI, SEJ, SEK, SEL, SEM, SEN, SEO
- SEs are heat stable, 26,900-29,600 Dalton, globular proteins rich in lysine, aspartate, glutamic acid and tyrosine
- Most frequently associated with foodborne intoxication: SEA, SEC, SED
- Prior report from Indonesia: of 20 the SE produced from milk isolates, the most frequently isolated toxin is SEC (30%) (Salasi et al., 2009)
Introduction

*Staphylococcus aureus* Food Poisoning

<table>
<thead>
<tr>
<th>Year</th>
<th>Location</th>
<th>Food</th>
<th>No. of cases</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1989</td>
<td>Starkville, MS, US</td>
<td>Can mushroom</td>
<td>22</td>
<td>CDC, 1989</td>
</tr>
<tr>
<td>1990</td>
<td>Hospital, Puerto Rico</td>
<td>Ham</td>
<td>NA</td>
<td>Bergdoll, 1992</td>
</tr>
<tr>
<td>1996</td>
<td>Germany</td>
<td>Schwarzwalden Schinken</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1997</td>
<td>FL, US</td>
<td>Ham</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>Japan</td>
<td>Pasteurized milk</td>
<td>14,555</td>
<td></td>
</tr>
<tr>
<td>2002</td>
<td>Australia</td>
<td>Rice, potato, beef</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td>2007</td>
<td>Padang, Indonesia</td>
<td>Sticky rice dish</td>
<td>36</td>
<td>Gentina et al., 2008</td>
</tr>
</tbody>
</table>

Objectives

- To identify the relatedness of *Staphylococcus aureus* previously isolated from coconut rice and shredded chicken using Polymerase Chain Reaction (PCR)
- To detect the presence of enterotoxin genes for SEA and SEC in the local isolates using PCR
Methods

Microorganisms

- 14 local isolates *S. aureus* from shredded chicken (AS) and coconut rice (NU1, NU2, NU3, NU4, NU5, NU6, NU7, NU8, NU9, NU10, NU11, NU13, NU14 and a non-enterotoxigenic *S. aureus* ATCC 25923

Primers

- 63f/1387r encoding for universal 16S rRNA (Marchesi et al. 1998)
  
  CAG GCC TAA CAC ATG CAA GTC
  GGG CGG WGT GTA CAA GGC

- SEA-1/SEA-2 encoding for SEA (Johnson et al. 1991)
  
  TTG GAA ACG GTT AAA ACG AA
  GAA CCT TCC CAT CAA AAA CA

- SEC1-1/SEC1-2 encoding for SEC1 (Johnson et al. 1991)
  
  GAC ATA AAA GCT AGG AAT TT
  AAA TCG GAT TAA CAT TAT CC

Methods

- Isolation
  
  Bacterial DNA was extracted with phenol chloroform isoamylalcohol, lysozyme and proteinase K (Doyle and Doyle 1990 with modifications). DNA was measured for its quality by spectrophotometry and verified on agarose gel after EthBr staining

- Amplification
  
  Amplification of gene encoding for universal 16S rRNA was done using a primer pair 63f and 1387r (Marchesi et al., 1998). The PCR protocol:
  
  pre-PCR (95°C, 3 min), denaturation (94°C, 30 sec), annealing (55°C, 30 sec), elongation (72°C, 1 min) and post-PCR (72°C, 5 min, 30. Visualization of the amplicon was observed by agarose (1.5%; w/v) gel electrophoresis at 50 V, 45 min

- Sequencing
  
  DNA fragment obtained from PCR was sequenced with ABI PRISM™ 3100-Avant 4-Capillary System Genetic Analyzer (Applied Biosystem), by PT. Macrogen Inc., Seoul, Korea Selatan. The resulted sequence was processed with BioEdit and analyzed using BLAST from NCBI at www.ncbi.nlm.nih.gov.
Methods

- Amplification of enterotoxin genes for SEA and SEC1 (Johnson et al., 1991).

**PCR for sea**
- (30 cycles)
- pre-PCR (95°C, 3 min)
- denaturation (94°C, 2 min)
- annealing (55°C, 90 sec)
- elongation (72°C, 1 min)
- post-PCR (72°C, 5 min)

**PCR for sec**
- (30 cycles)
- pre-PCR (95°C, 3 min)
- denaturation (94°C, 30 sec)
- annealing (54°C, 30 sec)
- elongation (72°C, 1 min)
- post-PCR (72°C, 5 min)

Visualization of the amplicons was observed by agarose (1.5%; w/v) gel electrophoresis at 70 V for 60 minutes.

Results

Visualization of amplified DNA fragment encoding for universal 16S rRNA using 63f/1387r primer pairs from local isolates of S. aureus dan S. aureus ATCC 25923 on 1.5% agarose gel: negative control (1), ATCC 25923 (2), AS (3), NU1 (4), NU2 (5), NU3 (6), NU4 (7), NU5 (8).

M is a 1 kb DNA ladder.
Results

Visualization of amplified DNA fragment encoding for universal 16S rRNA using 63f/1387r primer pairs from local isolates of *S. aureus* dan S. aureus ATCC 25923 on 1.5% agarose gel : ATCC 25923 (1), NU6 (2), NU7 (3), NU8 (4), NU9 (5), NU10 (6), NU11 (7), NU13 (8), NU14 (9). M is a 1 kb DNA ladder marker

Percent similarity of local isolates with several *S. aureus* from GenBank based on partial gene sequence of 16S rRNA

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Total score</th>
<th>Ref strain (source)</th>
<th>% similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS</td>
<td>239</td>
<td><em>S. aureus</em> subsp.aureus T0131 (clinical, China)</td>
<td>76%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>S. aureus</em> subsp.aureus str JKD6008 (clinical, Australia)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>S. aureus</em> subsp.aureus TW20 (clinical, London)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>S. aureus</em> subsp.aureus USA300 TCH1516 (clinical, US)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>S. aureus</em> subsp.aureus str Newman DNA (clinical, Japan)</td>
<td></td>
</tr>
</tbody>
</table>

| NU1     | 542         | *S. aureus* subsp.aureus T0131 (clinical, China) | 85           |
|         |             | *S. aureus* subsp.aureus str JKD6008 (clinical, Australia) |               |
|         |             | *S. aureus* subsp.aureus TW20 (clinical, London) |               |
|         |             | *S. aureus* subsp.aureus USA300 TCH1516 (clinical, US) |               |
|         |             | *S. aureus* subsp.aureus str Newman DNA (clinical, Japan) |               |
|         |             | *S. aureus* subsp.aureus NTCC 8325 (clinical,US) |               |
|         |             | *S. aureus* subsp.aureus USA300 FPR3757 (clinical, US) |               |
|         |             | *S. aureus* subsp.aureus clone sabac-1 (not known, US) |               |

| NU4     | 566         | *S. aureus* subsp.aureus ECT-R2 (human, Sweden) | 86           |
|         |             | *S. aureus* subsp.aureus ED98 (animal, US) |               |
|         |             | *S. aureus* subsp.aureus Mu3DNA (clinical, Japan) |               |
|         |             | *S. aureus* subsp.aureus JH1 (not known, US) |               |
|         |             | *S. aureus* subsp.aureus MU50 DNA (not known, Japan) |               |
### Percent similarity of local isolates with several *S. aureus* from GenBank based on partial gene sequence of 16S rRNA

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Total score</th>
<th>Ref strain (source)</th>
<th>% similarity</th>
</tr>
</thead>
</table>
| NU5     | 239         | *S. aureus* subsp. aureus ECT-R2 (human, Sweden)  
*S. aureus* subsp. aureus ED98 (animal, US)  
*S. aureus* subsp. aureus Mu3DNA (clinical, Japan)  
*S. aureus* subsp. aureus JH1 (not known, US)  
*S. aureus* subsp. aureus MUS0 DNA (not known, Japan) | 92           |
| NU9     | 865         | *S. aureus* subsp. aureus ECT-R2 (human, Sweden)  
*S. aureus* subsp. aureus ED98 (animal, US)  
*S. aureus* subsp. aureus Mu3DNA (clinical, Japan)  
*S. aureus* subsp. aureus JH1 (not known, US)  
*S. aureus* subsp. aureus MUS0 DNA (not known, Japan)  
*S. aureus* subsp. aureus T0131 (clinical, China) | 96           |
| ATCC 25923 | 375   | *S. aureus* subsp. aureus str JKD6008 (clinical, Australia)  
*S. aureus* subsp. aureus TW20 (clinical, London)  
*S. aureus* subsp. aureus USA300 TCH1516 (clinical, US)  
*S. aureus* subsp. aureus str Newman DNA (clinical, Japan)  
*S. aureus* subsp. aureus NTCC 8325 (clinical,US)  
*S. aureus* subsp. aureus USA300 FPR3737 (clinical, US) | 81           |

### Percent similarity between local isolates of *S. aureus* based on partial gene sequence of 16S rRNA

<table>
<thead>
<tr>
<th></th>
<th>A5</th>
<th>NU1</th>
<th>NU4</th>
<th>NU5</th>
<th>NU9</th>
<th>ATCC 25923</th>
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<tbody>
<tr>
<td>A5</td>
<td>100</td>
<td>76</td>
<td>-</td>
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<tr>
<td>NU1</td>
<td>100</td>
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<td>87</td>
<td>87</td>
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<td>NU4</td>
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<tr>
<td>NU9</td>
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<td>100</td>
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<tr>
<td>ATCC 25923</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100</td>
</tr>
</tbody>
</table>
Visualization of amplified DNA fragment encoding for *Staphylococcus enterotoxin A* (sea) using SEA-1/SEA-2 primer pair in local isolates of *S. aureus* dan *S. aureus* ATCC 25923 on 1.5% agarose gel

Negative control (1), ATCC 25923 (2), AS (3), NU1 (4), NU3 (5), NU4 (6), NU5 (7), NU6 (8), NU7 (9), NU8 (10), NU9 (11), NU11 (12), NU13 (13). M is a 1 kb DNA ladder marker

Visualization of amplified DNA fragment encoding for *Staphylococcus enterotoxin C1* (SEC1) using SEC1-1/SEC1-2 primer pair in local isolates of *S. aureus* dan *S. aureus* ATCC 25923 on 1.5% agarose gel

Negative control (1), ATCC 25923 (2), AS (3), NU1 (4), NU3 (5), NU4 (6), NU5 (7), NU6 (8), NU7 (9), NU8 (10), NU9 (11), NU11 (12), NU13 (13). M is a 1 kb DNA ladder marker
Conclusions .. (1)

- Five (AS, NU1, NU4, NU5, NU9) out of 12 of presumptive *S. aureus* isolated from RTE foods in Indonesia can be confirmed as *S. aureus* using primer for gene encoding for universal 16S rRNA.

- The five local isolates showed sequence similarity between 76 - 96% with other (mainly) human/clinical isolates.

- Similarity between the 5 local isolates is low (76-92%), needs to establish phylogenetic tree.

Conclusions .. (2)

- Of the 5 local isolates, NU1 was found to possess gene encoding for SEA, while NU5 has both genes for SEA and SEC1. However several isolates not confirmed by universal 16S rRNA seems to possess SEA or SEC1 gene (NU3, NU6,NU8), thus use of primer for universal 16S rRNA may not be adequate/efficient for molecular confirmation of *S. aureus*.
Acknowledgement

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Thank You

ratihde@ipb.ac.id
http://ratihde.staff.ipb.ac.id