Detection *Staphylococcus aureus* Producing Enterotoxin A on the Skewers Meatballs Product in Yogyakarta City Indonesia

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Abstract. Meatballs are snacks that are sold by street vendors in the school area and some public places in Yogyakarta city. Based on the materials used and the traditional producing process, it is possible that the hearty snacks can be contaminated by enterotoxin-producing *Staphylococcus* bacteria. This study aims to detect the presence of enterotoxin-producing *Staphylococcus* on skewers meatballs. Skewed meatball samples were taken from ten different locations in Yogyakarta city. The samples were grown on the *Staphylococcus* sp test standard medium, Baird Parker Agar (BPA). The colony candidates were then selected into Mannitol Salt Agar (MSA), carbohydrate fermentation medium, gram staining and then confirmed using the API Staph. Molecular characterization by detecting nuc genes and sea genes as encode enterotoxin A producers. The result of this research showed that the total bacteria ranged from $1.7 \times 10^4$ - $2.0 \times 10^9$ CFU/g. Identification results using API Staph were found as *S. aureus*, *S. cohnii*, *S. xylosus*, *S. lentus*, *S. warneri*, and *S. sciuri* while nuc and sea genes were only found in *S. aureus*.

1. Introduction

The *Staphylococcus* genus currently has more than 50 species and 28 sub-species i.e. gram-positive bacteria, with a diameter of 0.5-1.5 μm, its coci form is in grape like clusters, commonly living in colony on the skin or mucous membrane of human and other animals. This bacteria is naturally found as normal flora on animal skin and humans [9][10]. Most *Staphylococcus* sp bacteria are pathogenic and capable of producing toxins that can cause health problems for humans. Based on the findings of previous studies, this member of *Staphylococcus* sp genus that produces the most toxins and has caused health problems is *Staphylococcus aureus*. This bacteria mostly contaminates food, mainly due to traditional processing or dirty hand-contact. Contamination may also be caused by raw materials, equipment, containers, and poor environmental sanitation [7].

The previous studies had found *Staphylococcus* sp isolates in fresh milk that were sold by street vendors and cafes in Yogyakarta [12]. Evidently, *S. aureus*, *S. epidermidis*, and *S. hemoliticus* were also found in infant formula [19]. In addition to milk and its products, it is also found in home-scale snacks, i.e. the skewed meatballs in Bantul, Yogyakarta. The results of isolation and identification during the production process of skewed meatballs from raw materials preparation to the presentation were obtained by various kinds of *Staphylococcus* sp. Based on the confirmation results using API-STAP, several types of *Staphylococcus* were identified with the following determination index (% ID):
from the raw material, \textit{S. epidermidis} (97.6\%) and \textit{S. aureus} (97.9\%) were found, in the mixing process \textit{S. xylosus} (92.6 \%) was found; in kneading process isolates \textit{S. epidermidis} (97.1\%) and \textit{S. lentus} (99.8\%) were found, and in the presentation process \textit{S. lugdunensis} (74.7\%) and \textit{S. epidermidis} (97.1\%) were found [21]. \textit{Staphylococcus aureus}, \textit{S. epidermidis}, \textit{S. lentus}, and \textit{S. xylosus} isolates were also found in fruit and vegetable salads [22]. Skewers meatballs is the one of favorite snacks for many people in Yogyakarta. These meatballs are made using workers’ hands to allow staphylococcus contamination. This study aims to detect enterotoxin-producing \textit{S. aureus} in skewers meatballs sold in the school area and several public places in Yogyakarta and its surrounding areas.

2. Experimental

2.1. Samples

Skewer meatball samples were taken from 10 different locations which were often visited by the students and some public places which were crowded by costumers in Yogyakarta city and its surrounding areas. The samples were then transported to the Microbiology Laboratory of Universitas Kristen Duta Wacana for testing according to the standard method for the detection of \textit{Staphylococcus} sp.

2.2. Isolation and selection of \textit{Staphylococcus}

A sample of 10 grams was then mashed aseptically. It aimed to suspend all bacteria in 90 ml of 1\% peptone water medium. The cell cultures were grown into Baird Parker Agar (BPA) medium, incubated for 48 hours at 37 \degree C. The suspected colonies of \textit{Staphylococcus aureus} will give a blackish-gray appearance and have an opaque zone around the colony. The suspected colonies were then selected by streaking them into the Mannitol Salt Agar (MSA) medium and then incubating at 37\degree C for a maximum of 24 hours[1] [5][13]. The growth of \textit{S. aureus} colonies was marked by the color change of the colony to yellow in less than 24 hours. The colonies that turned yellow after 24 hours were non-aureus \textit{Staphylococcus} group. The yellow colonies were then isolated and grown into the Brain Heart Infusion (BHI) medium as a culture stock for the following tests, that being gran staining and carbohydrates fermentation. The biochemical test culture tubes were incubated at 37\degree C for 48 hours. The color change of the red medium to yellow indicated a positive result. Colonies that have been indicated to certain species of a \textit{Staphylococcus} sp genus were then separated to be subcultured into BHI Agar medium for 18-24 hours. It was then used as culture stock for the \textit{Staphylococcus} confirmation test using the API-Staph [17][18][24].

2.3. Biochemical identification of \textit{Staphylococcus}

The isolates were grown into the API-Staph medium, then homogenized and its turbidity levels were standardized using a 0.5\% McFarland standard solution. Furthermore, the cultures were dropped on biochemical test wells found in the Biomerieux Staph-API test kit. Mineral oil was then added into the wells used for ADH and UREA test to create an anaerobic condition. The incubation process was carried out at 37\degree C for 18-24 hours. After the incubation process, VP reagents (Voges Proskauer), PAL, NIT (nitrate) were added into wells used for the NIT, ZYM, and VP tests. Positive results of \textit{Staphylococcus} sp were confirmed using a positive control table and API web software (bioMerieux).

2.4. Molecular detection of \textit{nuc} and \textit{sea} genes

Each of the confirmed \textit{S.aureus} and non-aureus isolates from the API Staph test was taken for molecular testing by detecting \textit{nuc} and \textit{sea} genes. Molecular detection initiated with DNA isolation phase. The DNA isolates were tested using electrophoresis. The \textit{nuc} genes were amplified using reference primers [3][4][20]. It was performed using the following oligonucleotide sequences:
fw5’GCGATT GATGGTGATACGGTT-3’ and rv3’ GCCAAGCTTGCAGAACTAAAGC-5’. The reaction consisted of 1 μl ddH2O, 12.5 μl Mix PCR Promega, 1 μl of primer F, 1 μl of primer R, 1 μl DNA. The PCR phase of the nuc gene was amplified at 270 bp, pre-denatured 95°C for 2 minutes; 35 cycles of amplification (denaturation at 95°C for 60 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 60 seconds) and final extension at 72°C for 5 minutes. Sea gene was amplified using the following primers: (sea) - F5’- TTGGAAACGGTTAAAACGAA-3’ and (sea) R5’ GAACCTTCCCATCAAAAACA-3’ [14]. The reaction consisted of 1 μl of ddH2O, 12.5 μl of Mix PCR Promega, 1 μl of F Primer, 1 μl and R Primer, 1 μl of DNA. In PCR phase, sea genes were amplified at 120 bp, pre-denaturation was carried out for 3 minutes at 95°C, denaturation for 2 minutes at 94°C, annealing for 35 cycles for 1 minute 30 seconds at 58°C, and final extension for 5 minutes at 72°C.

3. Results and Discussion

3.1. Isolation and selection of Staphylococcus bacteria

The results of the growth of colonies of aureus and non-aureus Staphylococcus from the samples studied are shown in Figure 1. Colonies of S. aureus have a type of the selective medium of BPA. The BPA medium is a differential selective medium for the isolation and detection of Staphylococcus sp and S. aureus bacteria groups. BPA medium was chosen for the isolation of Staphylococcus sp in this study because the medium has been recommended by The International Organization for Standards, the Association of Official Analytical Chemists International (AOAC), Bacteriological Analytical Manual (BAM), American Public Health Association (APHA) and International Dairy Federation (IDF) [5].

![Typical colonies Staphylococcus on BPA medium](image)

**Figure 1.** Growing of Staphylococcus sp colonies on BPA medium.

The medium of BPA contained sodium pyruvate which played a role in stimulating the growth of S. aureus. The suspected S. aureus colonies that grew on the BPA medium have typical grayish black colonies due to tellurite reduction to tellurium. In addition the colonies also have haloed zones around the colony (opaque halo) due to lecithinase activity. The occurrence of lipolytic and egg yolk proteolytic formed a clear zone to appear around the colony. The opaque zone in the clear zone would only appear after 48 hours of incubation. In addition, the growth of S. aureus and S. xylosus in the BPA medium was also characterized by the presence of a clear zone that could be clearly seen in the medium, grayish black colonies. The BPA medium also contained lithium chloride and tellurite which
functioned to inhibit the growth of other microorganisms, whereas pyruvate and glycine selectively stimulated the growth of the Staphylococcus sp group [5][13][25].

3.2. Biochemical characteristics of Staphylococcus

At this stage, the suspected S. aureus colonies were selected into the MSA medium to confirm the suspicion because of their resistance to salinity. MSA medium contained beef extract and protease peptone so that it strongly supported the growth of Staphylococcus sp and other bacterial growths which would be inhibited by high salt content of 7.5%. Staphylococcus aureus would grow well on MSA medium because of its ability to ferment mannitol and resistance to salt which produced yellow colonies in less than 24 hours incubation. The group of coagulase-negative Staphylococcus and Micrococcus sp bacteria could not ferment mannitol, thus S. aureus has a very good opportunity to grow and it is easily separated from other bacteria. Yellow colonies isolated from the MSA medium were selected through gram staining [6] and carbohydrate fermentation tests. This test aimed to determine the ability of the suspected S. aureus isolates in fermenting sugar substrates in order to get the suitability of S.aureus properties based on identification keys of Staphylococcus sp (Bergey’s Manual of Systematic Bacteriology).

Staphylococcus aureus was able to change the color of the carbohydrate fermentation medium on the substrate maltose, trehalose, and sucrose while on the xylose substrate, the medium color did not change. Of all the samples were taken from 10 different locations, 25 isolates leading to S. aureus were discovered based on the appearance of gram staining which had the shape of cocci, purple-colored, and clustered. From the carbohydrate fermentation test, 10 suspected S. aureus isolates, 8 suspected S. xylosus, 2 isolates of S. lentus, and 3 isolates of S. epidermidis were found. All of which were confirmed biochemically using the API Staph (bioMerieux). The isolates were inoculated into the medium contained in the kit. They were then homogenized so that they had turbidity equivalent to 0.5 McFarland, then cell cultures were inoculated into the API Staph and incubated at 37°C for 24 hours. The biochemical test results indicated a change in the color of the reagent of the API Staph kit according to the bioMerieux procedure which gave positive and negative results. The test results were then inputted into the bioMerieux web API to find out the identification results of the bacteria tested. Of the 10 isolates tested, only 8 isolates had %ID≥70%, that being S. xylosus with 2 isolates, S. warneri with 1 isolate, S. sciuri with 1 isolate, S.cohni with 1 isolate and S. aureus with 2 isolates (Figure 2).

![Graph](image1.png)

**Figure 2.** The diversity of Staphylococcus sp contaminant on skewed meatballs.
3.3. Molecular detection of nuc and sea genes of Staphylococcus

All isolates identified as *S. aureus* were then molecularly characterized using *nuc* gene markers. The *nuc* gene is a marker for the thermonuclease enzyme that established to molecular identification of *S. aureus* [4], while the *sea* gene marker was used to determine the nature of enterotoxin. Detection of *nuc* and *sea* genes in *S. aureus* suspected isolates used *nuc1, nuc2* and *sea1, sea2* primers. The results of *nuc* and *sea* gene detection in *S. aureus* suspected isolates are shown in Figure 3.

![Figure 3. Detection nuc and sea genes of S. aureus and S. lentus.](image)

**Line 1.** Amplified fragment using primer for nuc gene (270 bp) of *S. aureus*; **2.** Marker; **3.** Amplified fragment using primer for sea gene (120 bp) of *S. aureus* and **4.** *S. lentus*.

Figure 3 shows that the DNA amplification process of *S. aureus* isolates was successfully carried out using *nuc1, nuc2* and *sea1, sea2* primers. The findings shows that the isolates identified as *S. aureus* based on the confirmation results using the API Staph with% ID 70% have *nuc* and *sea* genes. While the isolates identified as *S. lentus* showed no *nuc* gene, but had the *sea* gene. The presence of the *nuc* and *sea* genes in *S. aureus* indicates that these bacteria are high-heat resistant and able to produce enterotoxin A which may pose risk for human health [23]. *Staphylococcus aureus* strains that grow under different stress conditions have a higher resistance to heat than those grown in a physiological environment suitable for their growth [8]. In this study, it was found that not only *S. aureus* bacteria were contaminant bacteria in skewed meatballs, but also other *Staphylococcus* group bacteria such as *S. cohnii, S. warneri, S. lentus, S. xylosus*, and *S. sciuri*. *Staphylococcus cohnii*, *S. warneri, S. xylosus* is a group of *Staphylococcus* sp bacteria that also have opportunistic pathogenic properties [2]. *Staphylococcus lentus* is a commensal bacteria whose habitat is on the skin of various animal species, such as poultry and dairy animals. In addition workers on farms are also at risk as hosts of *S. lentus* bacteria [11]. Cases of infection caused by *S. lentus* bacteria are very rare in humans [15]. *Staphylococcus sciuri* is a bacteria that can be found in pets and domestic animals, both healthy and sick. This bacteria has a characteristic of surviving using inorganic salt as the only source of nitrogen. Similarly, *S. lentus* and *S. sciuri* also rarely causes infection in humans [16].

4. **Conclusion**
Meatball samples obtained from 10 different locations in Yogyakarta contained contaminants of *Staphylococcus* bacteria between $1.7 \times 10^4$ to $2.0 \times 10^6$ colonies/gram. The biochemical identification findings using the Staph API indicate that each skewed meatball sample has been contaminated by *S. aureus* bacteria with %ID of 70% and 92%. In addition, other types of *Staphylococcus* were also found, that being *S. lentus* with %ID of 99.6%, *S. xylosus* with %ID of 99.8%, *S. cohnii* with %ID of 96.6%, *S. sciuri* with %ID of 86% and *S. warneri* with %ID of 79.4%. Molecular identification findings on isolates identified as *S. aureus* with %ID of 70% in the Staph API confirmation test shows a positive result of having a nuc gene at 270 bp and sea gene at 120 bp. In addition to other *Staphylococcus*, *S. lentus* only has sea genes that have the potential to produce enterotoxins A.

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