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Characterization of Haemolysin of *Staphylococcus aureus* Isolated from Food of Animal Origin

Dwi Ariyanti[1], Siti Isrina Oktavia Salasia[2*], and Syarifudin Tato[3]

[1]Research Center for Biotechnology, Universitas Gadjah Mada, Yogyakarta, Indonesia
[2]Department of Clinical Pathology, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia
[3]Department of Pharmacology, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia

Abstract

Staphylococcus aureus is an important pathogen bacteria causing food poisoning and various infection in animals and humans. Haemolysin is one of the virulence factors of *Staphylococcus aureus*. The aims of the research were to characterize haemolysins of *Staphylococcus aureus* isolated from various food of animal origin, phenotypic- and genotypically. In the present study, eleven *Staphylococcus aureus* isolated from various food of animal origins from traditional markets and supermarkets in Yogyakarta, Sidoarjo, Jakarta, and Bandung were characterized for haemolysin, pheno- and genotypically. Characterization of haemolysin phenotypically based on haemolysis pattern of *Staphylococcus aureus* on sheep blood agar plate. Genes encoding hemolysin were amplified with specific primers by using *polymerase chain reaction* (PCR) technique. The results of the studies showed that *Staphylococcus aureus* on sheep blood agar plates revealed an alpha haemolysis pattern (18,18%), beta haemolysis (27,27%) and gamma haemolysis (54,55%). Based on amplification of the gene encoding haemolysin of *Staphylococcus aureus* with specific primers showed *hla* genes (81,81%), and *hla* combined with *hlb* genes (18,18%). The amplification of gene *hla* and *hlb* had a single amplicon with a size of approximately 534 bp and 833 bp, respectively. The haemolysin characteristics of *Staphylococcus aureus* from various food of animal origin could be used as important information to control staphylococcal food poisoning.

Keywords : *Staphylococcus aureus*, haemolysin, PCR, food of animal origins

Introduction

Staphylococcus aureus is well known as bacterial pathogen of both human and animal. In humans, this bacteria causes food poisoning, toxic shock and variety of pyogenic infections (Le Loir *et al*., 2003). *Staphylococcus aureus* also could cause mastitis in cows, sheeps and goats, leading to severe economic losses worldwide (Roberson *et al*., 1994; Salasia *et al*., 2004).

*)**Corresponding author:**
Siti Isrina Oktavia Salasia
Department of Clinical Pathology, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia E-mail: isrinasalasia@ugm.ac.id

The primary habitat of *S.aureus* is in the nasal passage on the skin and hair of human and warm-blooded animals. The transmission of the organisms may occur through skin lesions, contaminated food, including milk and other animal products (Le Loir *et al*., 2003; Boerema, *et al*., 2006).

Staphylococcus aureus is an important food-borne pathogen because of its ability to produce a wide range of extracellular protein toxins and virulence factors that contribute to pathogenecity of the organism (Dinges *et al*., 2000). The pathogenecity of *S. aureus*, is related to the production of a wide variety of exoproteins, including alpha and beta haemolysins which contributes to its

ability to cause diseases in many mammalian species (Da Silva, 2005).

Staphylococcal haemolysin are identified as an important virulence factor that contribute for bacterial invasion and to escape from the host immune response. Alpha-haemolysin or alpha toxin considered to be a main pathogenicity factor because of its haemolitic, dermonecrotic and neurotoxic effects. Additionally, beta-haemolysin contains sphingomyelinase that more active against sheep and bovine erythrocytes (Da Silva, 2005).

Several studies indicated that haemolysins of *S. aureus* correlated well with mastitis. However, there are only few informations about haemolysins of *S. aureus* isolated from food of animal origin. This study was designed to determine the haemolysin of *S. aureus* isolated from various foods of animal origin based on their pheno- and genotypic characters.

Materials and Methods

Bacterial isolates

A total number of 11 *S. aureus* isolated from foods of animal origin were used in the present study (Table 1). The samples were collected from foods of animal origin from various cities.

All isolates were identified as *S. aureus* based on Gram staining, coagulase and catalase test, and fermentation of mannitol salt agar (MSA). Molecular identification was done according to the amplification of 23S rRNA genes using PCR method. The program and primer design were performed as previously described (Straub *et al.*, 1999) (Table 2).

Table 1. The origin of samples

No	Code	Source	City
1	K1	Cheese	Sidoarjo
2	SR1	Packaged Milk	Yogyakarta
3	YK1	Fermented Milk	Yogyakarta
4	RO	Rollade	Yogyakarta
5	SS2	Sosis	Yogyakarta
6	RY6	Cake	Yogyakarta
7	B3	Meat ball	Yogyakarta
8	BL2	Cheese Bolen	Bandung
9	SY1	Packaged Milk	Yogyakarta
10	SI1	Packaged Milk	Yogyakarta
11	BW4	Brownies	Jakarta

Haemolysins characterization

Type of haemolysins were characterized based on the lysis zone of each *S. aureus* isolates on the blood agar plate after 24 h incubation at 37°C. The genes encoding for haemolysins of *S. aureus* were performed by amplification of *hla* genes for alpha haemolysin and *hlb* genes for beta haemolysins using PCR method. The PCR programs and the sequences of the primers are listed in Table 2.

Isolation of DNA

The bacterial DNA was prepared with the QIAamp tissue kit as described by the manufacturer (Qiagen, Hilden, Germany). After cultivation of the isolates for 24 h at 37°C on blood agar plates, 5-10 colonies of the bacteria were suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA (pH 8) containing 5 µl lysostaphin (1.8 U/µl; Sigma, St. Louis,

Table 2. Oligonucleotide primers for amplification of 23S rRNA and genes encoding haemolysin

Gene	Primer sequence	Program(*)	Reference
23S rRNA	5'AGC GAG TTA CAA AGG AGG AC 3' 3'AGC TCA GCC TTA ACG AGT AC 5'	1	Straub *et al.*, 1999
hla	5'GGT TTA GCC TGG CCT TC 3' 3'CAT CAC GAA CTC GTT CG 5'	2	Booth *et al.*, 2001
hlb	5'GCC AAA GCC GAA TCT AAG 3' 3'GCG ATA TAC ATC CCA TGG C 5'	2	Booth *et al.*, 2001

(*) 1 : 30 times 94°C-120 s, 55°C-120 s, 72°C-60 s; 2 : 30 times 94°C-30 s, 50°C-30 s, 72°C-60 s.

Table 3. Identification of *S. aureus*

Code	Food Sample	Source of Isolate	Gram Staining	Fermen-tation of MSA*	Cata-lase	Coa-gulase	23S rRNA Gene
K1	Cheese	Sidoarjo	+	+	+	+	+
SR1	Packaged Milk	Yogyakarta	+	+	+	+	+
YK1	Fermented Milk	Yogyakarta	+	+	+	+	+
RO	Rollade	Yogyakarta	+	+	+	+	+
SS2	Sosis	Yogyakarta	+	+	+	+	+
RY6	Cake	Yogyakarta	+	+	+	+	+
B3	Meat ball	Yogyakarta	+	+	+	+	+
BL2	Cheese Bolen	Bandung	+	+	+	+	+
SY1	Packaged Milk	Yogyakarta	+	+	+	+	+
SI1	Packaged Milk	Yogyakarta	+	+	+	+	+
BW4	Brownies	Jakarta	+	+	+	+	+

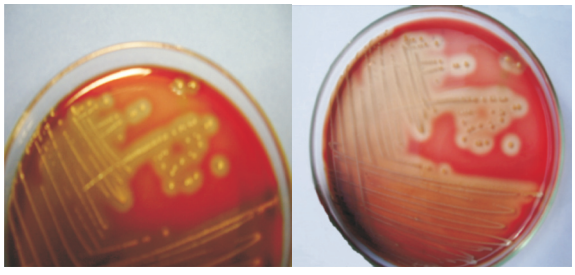
* MSA = mannitol salt agar

Missouri, USA). After 1 h incubation at 37°C, 25 µl of proteinase K (14,8 mg/ml; Sigma) and 200 µl of buffer AL (containing reagents AL1 and AL2) were added. The suspension was incubated for 30 min at 56°C and for 10 min at 95°C, and after a spin for a few seconds an amount of 420 µl ethanol was added to each sample and placed in a spin column. After centrifugation for 1 min, the QIAamp spin columns were placed in a clean collection tube and the samples were washed twice with 500 µl of buffer AW. After a second wash and a centrifugation for 3 min, the QIAamp spin columns were placed in a clean 2 ml microfuge tube and the DNA was twice eluted with 200 µl and 100 µl of buffer AE, respectively. The DNA could be stored at -20°C. Amplification reactions with PCR were perfomed by program and specific primer according to the references for 23S rRNA and *hla* and *hlb* genes. PCR products were then separated by gel electrophoresis in a 1.5% w/v agarose (Seakem) gel run in 0.5 × TBE buffer. The 1 kb Plus DNA ladder (Invitrogen, Netherlands) was used as a size marker. Resulting bands were visualized with cyber safe by UV transillumination.

Results and Discussion

According to Gram staining, biochemical properties and to the analysis of the PCR products of 23S rRNA specific region, all isolates in the present study could be identified as *S. aureus*. All cultures investigated were Gram positive, positive for coagulase and catalase, and fermented of MSA (Table 3). A comparable PCR-based system for identification of *S. aureus* isolated from various origins had already been used by numerous authors (Straub *et al.*, 1999; Annemüller *et al.*, 1999; Akineden *et al.*, 2001; Salasia *et al.*, 2004).

The types of haemolysins of *S. aureus* on the sheep blood agar plate, revealed alpha hemolysis for 2 isolates (18,18%), beta haemolysis for 3 isolates (27,27%) (Figure 1) and gamma hemolysis for 6 isolates (54,55%) (Table 4).



A B

Figure 1. Alpha-haemolysis (A) and beta-hemolysis (B) of *Staphylococcus aureus* isolates K1 and YK1 on the sheep blood agar plate.

Table 4. Characterization of haemolysins of *S. aureus*

Code	Food Sample	Source of Isolate	Haemolysis Type on Sheep Blood Agar Plate	*hla* gene	*hlb* gene
K1	Cheese	Sidoarjo	α	+	-
SR1	Packaged Milk	Yogyakarta	β	+	-
YK1	Fermented Milk	Yogyakarta	β	+	-
RO	Rollade	Yogyakarta	Γ	+	-
SS2	Sausage	Yogyakarta	γ	+	-
RY6	Cake	Yogyakarta	γ	+	-
B3	Meat ball	Yogyakarta	γ	+	-
BL2	Cheese Bolen	Bandung	γ	+	-
SY1	Packaged Milk	Yogyakarta	γ	+	-
SI1	Packaged Milk	Yogyakarta	α	+	+
BW4	Brownies	Jakarta	β	+	+

By PCR amplification of the gene encoding haemolysin of *S. aureus* with specific primers could be observed *hla* gene for 9 isolates (81,81%), and 2 isolates with two genes in combination *hla* and *hlb* (18,18%). The size of amplicon for *hla* gene was approximately 534 bp and 833 bp for *hlb* gene (Figure 2 and 3).

The *S. aureus* isolates with *hla* genes expressed alpha-, beta- and gamma-haemolysis on the sheep blood agar. The *S. aureus* with two genes in combination of *hla* and *hlb* expressed phenotypically as alpha- and beta-haemolysis. The genotypes of haemolysin of *S. aureus* in the recent study seemed to be not having correlation with the expression of their phenotypes. It might be influenced by many factors on the level of genetic or phenotypic. Blood collected from various animals on agar plates could show variable haemolysis pattern of *S. aureus*. The sheep blood shows an alpha, beta or gamma haemolysis of *S. aureus* on agar plates. However, the horse blood specifically showing a delta haemolysis of *S. aureus* on agar plates (Da Silva, 2005). Most of *S. aureus* isolated from human have usually an alpha haemolytic character, because the human platelets and monocytes are more sensitive to the alpha toxin. *S. aureus* isolated from animal are mostly characterized beta toxin because of the sensitivity of animal erythrocytes to this toxin (Todar, 2005). *S. aureus* isolated from foods

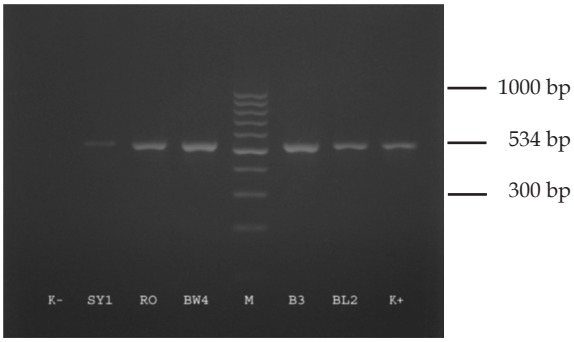


Figure 2. PCR Amplification of *hla* gene of *S. aureus* isolated from food, with the amplicon size of approximately 534 bp, in 2% of agarose gel electrophoresis. M= marker DNA ladder, K+/- = positive/negative control.

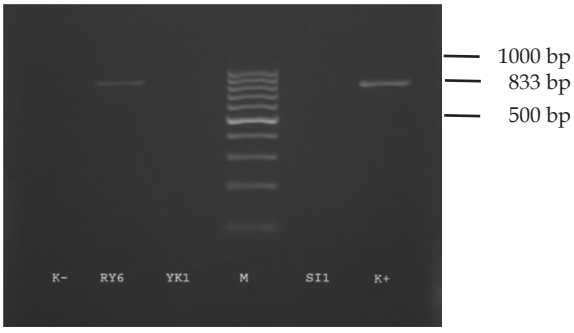


Figure 3. PCR amplification of *hlb* gene of *S. aureus* isolated from food, with the amplicon size of approximately 833 bp, in 2% of agarose gel electrophoresis. M= marker DNA ladder, K+/- = positive/negative control.

used in this study might have more complex haemolysin characters. Cross contamination between humans and food might occur via humans with skin lesions or via nasal discharge (Sandel and Mc. Killip, 2002). Food were probably affected by food contamination during handling or food processing. The temperature plays also an important role to the haemolysis character and in the mechanism of *hot cold lysis* for beta toxin (Todar, 2005). The sheep blood agar used in this study could show alpha, beta and gamma haemolytics, nor delta hemolytic. The age of the sheep was also reported influence to the type of haemolytic of *S. aureus*. In this study was used blood from a sheep with age of about 1 year old, that might contain of haemoglobin with the concentration higher than the younger (Mitruka and Rawnsley, 1981). Agar plate that contain old sheep blood may cause a dark back ground of plates that can confusing on the haemolysis examination. *S. aureus* with *hla* gene should express an alpha haemolytic. However, in this research some isolates revealed gamma haemolytic on blood sheep agar plate. It is quite possible the haemolytic type were influenced by high concentration of haemoglobin on sheep blood.

The existence of *hla* and *hlb* genes in *S. aureus* isolates are important for this strains, related to staphylococcal infection cases that caused food poisoning. This study demonstrated that the *hla* and *hlb* genes widely distributed among *S. aureus* isolated from food of animal origins. Indeed, although their true incidence in staphylococcal infection cases that caused food poisoning has still need to be clarified, it is now thought likely that their role has been underestimated.

In conclusions, the variable pattern of haemolysins of *S. aureus* established by the characteristic properties of samples which were highly determined by contamination both of animal product (milk, meat, and egg) as the basic material, or human as a food processor. Environment was also plays an important role for the hygiene and sanitation of the food process and storage. The haemolysin characteristics of *S. aureus* from various food of animal origin could be used as important information to control staphylococcal food poisoning.

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