Keberadaan Protein A pada Permukaan Sel Bakteri *Staphylococcus Aureus* Menggunakan Teknik "Serum Soft Agar"

The Presence of Protein A on The Surface of Staphylococcus Aureus Bacterial Cells Using Serum Soft Agar Techniques

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ABSTRAK

Protein A merupakan komponen protein spesifik di permukaan Staphylococcus aureus (S. aureus). Bakteri yang memiliki protein A banyak dimanfaatkan dalam biomedis. Penelitian ini bertujuan untuk menemukan metode sederhana mendeteksi keberadaan protein A pada S aureus.

Penelitian menggunakan 15 isolat S. aureus dari kasus lapangan, S. aureus cowan I sebagai kontrol positif, Staphylococcus epidermidis dan Micrococcus sp sebagai kontrol negatif. Bacteria diisolasi pada 10 ml medium Todd Hewith Broth, medium soft agar (SA), dan pada 10 ml serum soft-agar/SSA (SA yang ditambahkan serum kelinci atau ayam), agitasi menggunakan vortex dan diinkubasi pada 37^{o}C selama 18-24 jam. Bentuk koloni yang tumbuh diamati dan dikategorikan sebagai koloni kompak dan difus. Koloni yang diduga mengandung protein A dilakukan konfirmasi tes dengan Dot Blot.

7 isolat S. aureus menunjukkan perubahan koloni dari difus menjadi kompak pada SSA yang mengandung serum kelinci, dan 8 isolat tetap difus. Tes konfirmasi Dot Blot positif. Protein A diketahui mampu berikatan dengan fraksi Fc-IgG tetapi tidak dengan IgY unggas. Ikatan S. aureus dengan Fc-IgG dapat ditampilkan dengan perubahan bentuk koloni, dari difus sebelum penambahan serum, menjadi kompak setelah penambahan serum kelinci. Ikatan antara protein A dengan IgG pada permukaan sel bakteri menyebabkan hambatan pertumbuhan dan ini terekspresi sebagai bentuk koloni kompak pada SSA (IgG). SAA mengandung serum mamalia dapat digunakan untuk membedakan bakteri yang mengandung atau tidak mengandung protein A di permukaan selnya.

ABSTRACT *Protein A is a specific proteins on the surface of some Staphylococcus aureus (S. aureus) strains. Staphylococcus bacteria which have protein A has widely used in the biomedical. Aims of this study to find a simple method of detecting the presence of S. aureus protein A.*

15 S. aureus isolates from field cases, S. aureus cowan I as positive control, Staphylococcus epidermidis dan Micrococcus sp as negatif control were used. Bacteria was inoculated into 10 ml Todd Hewith Broth medium, Soft Agar (SA) and into 10 ml of serum soft-agar (SSA), agitated using a vortex and incubated at 37^{o}C for 18-24 h. Form of colonies that grew was observed and categorized as compact and diffuse colonies. The colonies that suspected contain protein A conducted confirmation tests by Dot Blot.

7 isolates showed the change of colony formation from diffuse to compact in SSA (SA added rabbit or chicken serum), and 8 isolates remain diffuse. Dot Blot test positive. Protein A has the ability to bind the Fc fraction of IgG but not the IgY from birds. The binding of S. aureus and IgG could be demonstrated by the change of colony growth from diffuse before the addition of serum containing IgG in SA to compact after the presence of serum. Protein A on the bacterial cell surface bind the Fc-IgG caused steric hinderance of bacteria and expressed as compact colony formation in SA. SSA using mammalian sera can be used to discriminate the bacterial strains with or without protein A.

Staphylococcus bacteria which have protein A has a very important benefit in the biomedical world, because of its use in the preparation of a diagnostic kit, the purification of biological material such as immunoglobulins, antigens, enzymes, hormones and other important substances. Therefore, it is necessary to look for the *Staphylococcus aureus* (*S. aureus*) or any other staphylococcus bacteria type, which has and has rich a protein A. Currently, Currently, *S. aureus* Cowan I is known as a rich protein A *S. aureus* strain.

The protein A containing on the bacteria surface can be reduced by the getting older isolate's age and because the isolates have been passage repeatedly. The use of serum soft agar technique in determination of the streptococcus B group serotype and in determination of the presence of cα and cß proteins of *Streptococcus agalactiae* have also been reported (Wibawan and Laemmler, 1990[a]; Wibawan and Laemmler, 1990[b]) and it was used to differentiate encapsulated and not encapsulated staphylococcus bacteria which isolated from human clinical cases and also cow mastitis patients (Opdebeeck *et al*., 1985, Opdebeeck *et al*., 1987, Opdebeeck *et al*., 1988). The serum soft agar technique has also been used to determine the serotypes of *Staphylococcus epidermidis*, using specific serum against certain serotypes candidate. Homologous *S. epidermidis* strains will show changes from diffuse to compact colonies form, while heterologous strains will remain diffuse colonized (Yoshida *et al*., 1972).

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Protein A as biologicaly acts has a role as a bacterial virulence factor (DeDent *et al*., 2007; Yao *et al*., 2010) that is capable to bind the Fc fraction of almost all immunoglobulin G subclasses except IgG3 (human), IgG1 (mouse), IgG1, IgG2a , IgG2b (rat), as well as chicken immunoglobulin Fc (Djannatun, 2002).

The interaction of IgG with protein A using a variety methods have been reported. They are a double gel immunodiffusion method, immunofluoresensi, indirect hemagglutination, ELISA, slide hemagglutination test, microplate hemagglutination test (Huang and Chang, 2004; Cox *et al.,* 1986; Takeuchi *et al.,* 1988, Takeuchi *et al*., 1995), single radial immunodiffusion, solid-phase radio immunoassay (Cheung *et al*., 1987), PCR (Wenwen *et al*., 2016). In this study showed the using of serum soft agar technique using rabbit serum to differentiate *S. aureus* that has and do not has protein A.

MATERIALS AND METHODS

Time and place

This research was conducted in March 2000 until November 2001 in Fakultas Kedokteran Hewan Institut Pertanian Bogor and Biofarma bandung.

Bacterial Isolated

In this study, 15 *Staphylococcus aureus* isolates from field cases were used. All isolates had been identified previously as *S. aureus*, and existed as a collection of Fakultas Kedokteran Hewan Institut Pertanian Bogor. *Staphylococcus aureus* Cowan I, which has rich protein A, and *Staphylococcus epidermidis*, which has no protein A, used as reference strains.

Reidentification of *S. aureus* Isolates

All *S. aureus* isolates was reidentificated using standard procedures for these bacteria, that are the type of colony formation and type of haemolysis on blood agar medium, shape and composition of bacterial cells microscopically with Gram stain (Carter, 1986), and the ability to utilize glucose and mannitol and the expression of catalase and coagulase activities (Qian *et al*., 2007).

The soft agar technique

The *S. aureus* bacteria was inoculated into 10 ml *Todd Hewith Broth* (THB) medium at 37^0C for 18-24 h, then Soft Agar (SA) was made consisting of 0.15% agar base in 10ml of Brain Heart Infusion medium (BHI). SA was boiled on the water until the liquid homogeneous, while boiling. After that it was cooled to about 37-40^{o}C temperature.

The suspension of bacteria in media THB was taken with ½ cm oese needle, then was inoculated into 10ml NaCl physiological sterile saline. The solution and then was homogenized so that bacterial cells were spread evenly and bacterial suspension (1/2 cm oese needle) was inoculated into 10 ml of *soft-agar*, agitated using a vortex and incubated at 37^{o}C for 18-24 h. Form of colonies that grew observed and categorized as compact colonies and colonies diffuse Wibawan & Lämmler, 1990^a; Wibawan & Lämmler, 1990^b).

Bacteria Growth in Serum Soft Agar (SSA)

This method was used to find the bacteria containing protein A and to show its ability to interact with the rabbits immunoglobulin (IgG) and several serum from avian species. For this purpose soft agar was made so as

has been described, and then added 100 ul of rabbit serum or other avian serum. Bacterial suspension (1/2cm oese needle) was inoculated into 10 ml of serum soft-agar, agitated using a vortex and incubated at 37^{o}C for 18-24 h. Form of colonies that grew was observed and categorized as compact colonies and colonies diffuse as well as in SA (Wibawan dan Laemmler, 1990[b]).

Dot Blot

The presence of protein-A on the cell surface of *S. aureus* bacteria was confirmed with the dot blot test. Isolates of *S. aureus* Cowan I, Sa53, 2 *S. aureus* selected isolates that showed a compact colonies in SSA and *S. epidermidis* were dropped onto a nitrocellulose membrane, dried with hair dryer. After that a nitrocellulose membrane submerged in skimmed milk which had been diluted 10 times for 45 minutes at room temperature, washed with 5 ml PBS (pH 7,5) 2x, and incubated with rabbit serum or chicken serum each for an hour followed by 2x washing with 5ml PBS. Incubated again in mice- anti-rabbit conjugate or mice-anti-chicken (25 µl conjugates + 5 ml PBS solution) for 60 min, followed by washing with PBS solution. The interaction of protein-A and IgG was visualized by adding 5ml of α-chloronapthol (9ml α-chloronapthol + 3 ml metanol + 25ml PBS) and 200 µl of H_2O_2. Positive reaction was characterized by the presence of black color on bacterial suspension droplets which were tested (Wibawan, 1993).

RESULTS

Characterization of *S. aureus*

Staphylococcus aureus isolates which were grown on blood agar medium was studied through its colony characteristic and its growth characteristic on BHI (*Brain Hearth Infusion*) medium after incubated at 35 ± 1^{0} C for 24-48 h. Besides that, the morphology and composition of bacterial cells microscopically was studied by Gram staining. For identification (determination of species) was studied by catalase test, coagulase and oxidase test as well as the ability of bacteria to fermentation mannitol. The *S. epidermidis* and micrococcus bacteria used as a comparison, as bacteria, that have no protein A.

Staphylococcus aureus isolates which were grown on blood agar media showed colony characteristics with a flat edge convex, shiny, smooth, with a 0.8-1.0m in diameter, produces a wide zone with complete hemolysis (β hemolysis). All *S. aureus* isolates which were used were aerobikal mesophyll, grew well after incubated in BHI medium at 35 ± 1^{0} C for 24-48 h. All isolates were spherical (cocci), arranged like grapes, Gram positive in Gram staining, aerobic and facultative aerobic, had catalase, coagulase and oxidase activities and mannitol fermented, positive coagulase (Cowan and Steel, 1973; Hof and Dörries, 2002; Brooks *et al*., 2010; Bien *et al*., 2011; Fehrmanna *et al*., 2013). (Table 1).

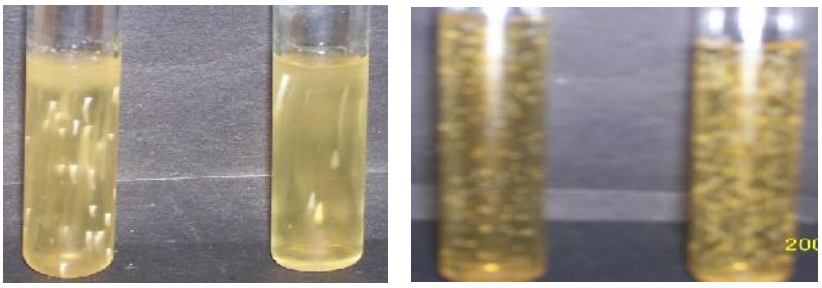
Table 1. Characteristic of *S. aureus* bacteria in some tests compared with *S.epidermidis* dan *Micrococccus sp.*

Confirmation tests	Characterictic		
	S. aureus	***S. epidermidis***	***Micrococcus sp***
Gram staining	Spherical shape, arranged like grapes, Gram positive	Spherical shape, arranged like grapes, Gram positive	Spherical shape, arranged like grapes, Gram positive
Catalase activity	+[a]	+[a]	+[a]
Coagulase	+[b]	-[a]	-[a]
Anaerobic utilization of:			
Glucose	+[c]	+[c]	-[b]
Mannitol	+[d]	-[c]	-[c]

Explanation: +[a] = produce air bubbles, +[b] = rabbit plasma agglutinate, -[a] = non rabbit plasma agglutinate, +[c] = glucose fermented, -[b] = non glucuse fermented, +[d] = mannitol fermented, -[c] = non mannitol fermented.

Determination of the Rich Protein A *S. aureus*

To determine *S. aureus* isolate containing rich protein A, Serum soft agar technique was used. The changes of colony formation from diffuse to compact are indicators of the presence of protein A on the bacterial cell surface. Isolat S.a-3, S.a-4, S.a-5, S.a-6, S.a-11, S.a-53, S.a-54 and S.a Cowan I showed the change of colony formation from diffuse to compact colonies in SSA, while others *S. aureus* isolates showed remain diffuse colonies in SSA. Serum soft agar (SSA) using rabbit serum were able to distinguish groups of *S. aureus* isolates compact colonized and diffuse colonized in SSA (Table 2 and figure 1).



Diffuse colony form Compact colony form

Figure 1. The changes of colony diffuse (before addition of rabbit serum) to compact (after addition of rabbit serum) in *S. aureus* which has protein A with serum soft-agar technique.

Table 2. Detection the presence of protein A on the surface of *Staphylococcus aureus* bacterial cells using serum soft-agar.

Name/Code of culture	Colony Form			Explanation
	No Serum	Rabbit serum	Chicken serum	
S.a -1	Diffuse	Diffuse	Diffuse	Dot blot tested
S.a -2	Diffuse	Diffuse	Diffuse	
S.a -3	Diffuse	Compact	Diffuse	
S.a -4	Diffuse	Compact	Diffuse	
S.a -5	Diffuse	Compact	Diffuse	
S.a -6	Diffuse	Compact	Diffuse	
S.a -7	Diffuse	Diffuse	Diffuse	
S.a -8	Diffuse	Diffuse	Diffuse	
S.a -9	Diffuse	Diffuse	Diffuse	
S.a -10	Diffuse	Diffuse	Diffuse	
S.a -11	Diffuse	Compact	Diffuse	
S.a -12	Diffuse	Diffuse	Diffuse	
S.a -13	Diffuse	Diffuse	Diffuse	
S.a-53	Diffuse	Compact	Diffuse	Dot blot tested
S.a-54	Diffuse	Compact	Diffuse	Dot blot tested
S.a-Cowan I (Ref +)	Diffuse	Compact	Diffuse	Dot blot tested
S epidermidis (Ref -)	Diffuse	Diffuse	Diffuse	Dot blot tested

Explanation: S.a = *Staphylococcus aureus*

Furthermore, based on the quality of the colonies form changes were selected S.a-53 and S.a-54 to be confirmed the presence of protein A on the bacteria cells surface used Dot Blot assay (Table 3). The Dot blot assay showed the interaction of S.a-53 and S.a-54 isolates with rabbit IgG. It were characterized by the presence of black color on paper nitrselulose that were dropped two bacterial suspension. The same reaction was shown also by S.a Cowan I, while S.a-1 and *S. epidermidis* showed no reaction (Table 3). The S.a-53 and S.a-54 isolates showed the presence of protein A and S.a-1 showed no protein A. The results indicate that bacteria have a protein A have Dot Blot positive reaction. Otherwise bacteria that do not have protein A have Dot blot negative reaction. These results indicated also in bacteria reference, *Staphylococcus aureus* Cowan I, which has rich protein A, and *Staphylococcus epidermidis*, which has no protein A.

Table 3. A positive reaction to the dot blot assay relating to compact colonies form on serum soft agar (SSA)

Isolate	Dot Blot Reaction	Colony Form (SSA)
S.a-53	+	Compact
S.a-54	+	Compact
S.a Cowan I	+	Compact
S.a-1	-	Diffuse
S. epidermidis	-	Diffuse

Explanation: S.a = *Staphylococcus aureus*, + = has protein A, - = No protein A

DISCUSSION

S. aureus Characterisation

Based on the results of biochemical tests obtained 12 strains of *Staphylococcus aureus* (Cowan and Steel, 1973; Hof and Dörries, 2002; Brooks *et al*., 2010; Bien *et al*., 2011; Fehrmanna *et al*., 2013). The *S. epidermidis* and micrococcus bacteria used as a comparison and a differentiator other cocci-shaped bacteria (Table 1).

Detection of The presence of The Rich of Protein A *S. aureus*

From 15 *S. aureus* samples of fields isolates, 7 isolates showed the change of colony formation from diffuse to compact colonies after the presence of rabbit sera in serum soft agar (SSA), similar result was found by The reference using *S. aureus* Cowan I strain. No comparable results were found for 8 isolates and for *S. epidermidis* as a negative control strain, the colonies remained diffuse after the addition of rabbit sera in SSA. No change of colony formation was detected after the addition of chicken sera for all bacteria isolates used in this study (Table 2, figure 1).

The SAA technique can be used to discriminate the *S. aureus* bacterial strains with or without protein A on the bacteria surface cell. The results in Table 2 show that SSA using rabbit serum were able to distinguish groups of *S. aureus* isolates compact colonized and diffuse colonized in SSA. The changes of colonies form in this interaction was not caused by coagulase reaction or clumping factor because all *S. aureus* isolates, which were used in this study, had positive reaction in coagulase test. The change of colony formation was related with the interaction between a protein on the surface of bacterial cells with IgG in the rabbit sera. It was confirmed by the addition of chicken serum on soft agar not to cause a change in the bacterial colony formation.

According the scientific information which had been presented by previous researcher, protein A has the ability to bind the Fc fraction of Immunoglobulin G (IgG) but not the immunoglobulin Y (IgY) from chicken (Wen Wen *et al*., 2016; Larson *et al*., 1993). The results of this study confirmed and can be demonstrated the simple methode to show the ability of interaction IgG to protein A as one of the surface components of the cell wall of bacteria and at the same time also showed that IgY was not able to bind to protein A. For Further, based on the quality changes in the form colonies, selected S.a-53 and S.a-54 isolates to be tested using the Dot blot assay. The results of Dot Blot assay indicate that

bacteria have a protein A have Dot Blot positive reaction. Otherwise bacteria that do not have protein A have Dot blot negative reaction. These results indicated also in bacteria reference, *Staphylococcus aureus* Cowan I, which has rich protein A, and *Staphylococcus epidermidis*, which has no protein A.

SSA is a practical, simple and inexpensive method to detect the presence of protein A *Staphylococcus aureus* as compared to PCR and Elisa methode (Huang and Chang, 2004; Wenwen *et al*., 2016).

CONCLUSION

The SSA Technique using rabbit serum can be used to determine the presence of protein A on the surface of *Staphylococcus aureus* bacterial cell through the changes of the colony form from diffuse to compact. Immunoglobulin Y (IgY) chicken was not able interact with protein A of *S. aureus*.

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