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Simple Method for the Detection of Protein A positive *Staphylococcus aureus* for Anisakidae Rapid Test

(METODE DETEKSI SEDERHANA STAPHYLOCOCCUS AUREUS POSITIF PROTEIN-A UNTUK UJI CEPAT ANISAKIDAE)

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Abstract

Based on the unique properties of protein A which naturally binds to the Fc IgG fraction of mammals but not to the Fc IgY fraction of chickens. The serum-soft agar (SSA) technique can be used to sort *Staphylococcus aureus* isolates that have protein A from those that do not. Protein A-positive *S. aureus* will form compact colonies in SSA with rabbit serum, while those that do not will form diffuse colonies. The use of chicken serum in SSA will produce diffuse colonies, even when *S. aureus* has protein A. The compact colony reflects the inhibition of bacterial growth in SSA. Such a phenomenon might be related to the formation of interaction complexes between IgG and protein A on the surface of the bacteria, in which these complexes inhibit bacterial growth. No comparable results were shown by the protein A negative *S. aureus*. The presence of protein A on the surface of bacterial cells was confirmed by the occurrence of *Spa* gene using polymerase chain reactions (PCR). An isolate of protein A-positive *S. aureus* was chosen and coated with rabbit IgG specific against Anisakidae antigen to be used as a non-soluble matrix for the preparation of the Anisakidae co-agglutination rapid detection test.

Keywords: Protein A+ *Staphylococcus aureus*; Serum-Soft Agar; Anisakidae, Co-agglutination test

Abstrak

Berdasarkan sifat khas protein A yang secara alami mampu berikatan dengan fraksi Fc IgG mamalia tetapi tidak mampu mengikat fraksi Fc IgY ayam. Teknik serum-soft agar (SSA) dapat digunakan untuk memilah isolat *Staphylococcus aureus* yang memiliki protein A dengan yang tidak memiliki protein tersebut. Bakteri *S. aureus* yang memiliki protein A akan tumbuh dengan koloni berbentuk kompak dalam SSA menggunakan serum kelinci, sedangkan yang tidak akan tumbuh dengan bentuk koloni difus. Penggunaan serum ayam pada SSA akan menghasilkan koloni difus, meskipun *S. aureus* yang memiliki protein A. Bentuk koloni kompak mencerminkan terjadinya hambatan pertumbuhan bakteri di dalam SSA. Hal ini mungkin berkaitan dengan terbentuknya kompleks interaksi antara IgG dengan protein A yang ada di permukaan bakteri tersebut. Ikatan kompleks ini mengakibatkan hambatan pertumbuhan bakteri. Sebaliknya, tidak terjadi hambatan pertumbuhan bakteri *S. aureus* yang tidak memiliki protein A. Keberadaan protein A dikonfirmasi secara tidak langsung dengan melacak keberadaan gen *Spa* (gen penyandi protein A) menggunakan polymerase chain reactions (PCR). Satu isolat *S. aureus* yang memiliki protein dipilih dan dilapisi dengan IgG kelinci spesifik terhadap antigen Anisakidae dan digunakan sebagai matriks dalam pembuatan kit diagnostik koaglutinasi Anisakidae.

Kata-kata kunci: protein A+ *Staphylococcus aureus*; serum-soft agar; Anisakidae; co-agglutination test

INTRODUCTION

The Protein A-rich *Staphylococcus aureus* is commonly used as the non-soluble matrix in co-agglutination tests. The specific immunoglobulin-G (IgG) is cou-pled with the vegetative form of inactivated *S. aureus* through the interaction between cell-associated protein A and Fc-fraction of specific IgG. This method is simple and fast, as the results can be observed within 3-5 seconds. The co-agglutination test using homolog antibody-coated protein A-positive *S. aureus* is commonly used as a rapid test for antigen detection (Wibawan *et al.,* 2009).

The important character of protein A is its natural ability to bind to the Fc fraction of mammalian IgG, but not to the Fc fraction of chicken IgY (Larsson *et al.,* 1992). Based on this character, the presence of protein A on the surface of *S. aureus* cells might be detected by serum-soft agar method using rabbit sera. The specific interaction of Fc IgG with protein A on the surface of *S. aureus* bacterial cells might cause the inhibition of bacterial growth. The growth inhibition of bacteria could be demonstrated by the change of bacterial colony formation in a serum-soft agar (SSA) medium.

The change of colony formation was observed in the growth of group B streptococci in serum-soft agar containing specific sera of rabbit. The results of serotyping using co-agglutination were further confirmed with polymerase chain reaction and showed the same results. This indicated that the serum-soft-agar technique could be applied for serotyping of strains of group B streptococci. (Yao *et al.*, 2013). Serum-soft agar was used to distinguish the presence of the capsule of *S. aureus*. Encapsulation was assessed by the production of a diffuse colony in serum-soft agar. The presence of a capsule might block the interaction between surface antigens of bacteria with the colony-forming factors in mammalian sera. Based on this evidence, it is believed that the natural binding of Fc-IgG in rabbit sera with protein A-positive *S. aureus* caused the change in colony formation of the bacteria. Contrary to this, no changes in colony formation will be

observed in protein A negative *S. aureus*. The use of chicken sera in SSA will not cause the change of colony formation of protein A-positive *S. aureus* due to the lack of ability of Fc IgY to bind the protein A.

In this study, we detect the presence of protein A on the surface bacterial cells of *S. aureus* using a simple method and confirmed indirectly by the presence of *Spa* gene using PCR technique. Protein A-positive *S. aureus* then was chosen and used as the non-soluble matrix for the preparation of the rapid diagnostic test for Anisakidae.

RESEARCH METHODS

Bacterial Cultures

Eleven isolates of *S. aureus* were used in this study, namely eight isolates from clinically mastitis cases (SA IPB, SA Boyolali, SA-A1, SA-A2, SA-C1, SA-C5, SA-C6, and SA-E1), *S. aureus* Cowan 1 isolate as the positive control, and two isolates of *S. epidermidis* (NFW and NTC) as the negative control. All isolates were maintained in blood agar plates and stored at 4 °C.

Soft Agar (SA) and Serum-Soft Agar (SSA) Techniques

The soft agar medium contained Brain Heart Infusion (BHI, Gibco) and 0.15% agar. After inoculation of bacteria, the soft agar was incubated at 37 °C for 18-24 h. For the serum-soft agar, 100 µL of respective serum was added into the serum-soft agar, and respective bacteria were inoculated into SA.

Polymerase Chain Reaction for *Spa* gene of A Protein

The PCR amplification was performed in a thermocycler using gene sequences of primers of *Spa* gene (5` CAAGCACCAAAAGAGGAA – 3` and 5' CACCAGGTTTAACGACAT – 3`). A total of 37 PCR cycles were performed under the following conditions. Pre-denaturation of DNA at 95 °C for two min, denaturation of DNA at 94 °C for one min, primer annealing at 55.2 °C for one min, extension at 70 °C for one min, and final extension at 72 °C for five min. The PCR-amplified samples were analyzed by a horizontal agar gel electrophoresis (0.13% agar) (Yunita *et al.,* 2020).

Preparation of Coagglutination Test for Anisakidae

Co-agglutination tests were prepared according to the method described by Arnfia *et al*. (2017). Preparation of protein A-positive *S. aureus* (SA-IPB) was grown in brain heart usion (BHI) broth at 37 °C for 18-24 h, and harvested bacteria were washed twice with phosphate-buffered Saline (PBS), pH 7.2. The bacteria were then heat-inactivated at 60 °C for two h, adjusted to a concentration of 10% (v/v), and stored at 4 °C until used. As many as 100 µL of specific Anisakidae rabbit serum was mixed with 350 µL *S, aureus* suspension for Ttwo h at room temperature, two times washed with 0.5 mL of PBS, and finally resuspended to 0,40 mL of PBS + 0,10% Tween. Two drops of L3 WWE antigen were taken on a microscope slide, and an equal volume of co-agglutination reagent was added and mixed thoroughly. The slide was observed for two minutes for the appearance of a clear and distinct agglutination reaction.

RESULTS AND DISCUSSION

Protein-A Positive *Staphylococcus aureus* Differentiation in SSA

All of the *S. aureus* isolates used in this study expressed diffuse colony formations in soft agar media and serum-soft agar media using chicken serum (SSA+IgY) and four isolates (SA Cowan-1, SA-Boyolali, SA-1, and SA-C1) expressed compact colony formations in serum-soft agar using rabbit serum (SSA+IgG) (Tabel 1 and Figure 1). A can bind with strong affinity to the Fc portion of rabbit IgG but cannot bind to the Fc portion of chicken IgY (Pereira *et al.*, 2019).

The change of colony formation of *S. aureus* was shown by four isolates (SA-

Cowan 1, SA-Boyolali, SA-1, and SA-C1) from diffuse to compact in rabbit serum-soft agar. Contrary to this, no change in the colony formation of these four isolates in SSA with chicken sera (Table 1). These might be related to the presence of protein A on the surface of those respective bacterial cells. Protein A is a 42 kDa surface protein originally found in the cell wall of the bacteria *S. aureus*. This protein is encoded by the *Spa* gene. The interaction of the Fc fraction of rabbit IgG with Protein A might inhibit the growth of protein A-positive bacteria, but no comparable results were observed when the chicken sera were used. This indicated that there is no interaction between protein A on the surface of *S. aureus* and Fc-IgY in chicken sera. These results confirmed that the natural character of protein A could not bind the Fc-IgY (Kota *et al.*, 2019). No change in colony formation was shown by seven other isolates. The compact colony formation of *S. aureus* in serum-soft agar indicated the ability of protein A to bind Fc-part of immunoglobulin G and not a clumping factor-fibrinogen reaction (Forsum *et al.*, 1972).

Table 1. Colony formations of *S. aureus* in soft agar and serum-soft agar using chicken serum as well as rabbit serum.

No	Isolates	Soft Agar	Chicken Serum-Soft Agar (IgY)	Rabbit Serum-Soft Agar (IgG)
1	SA Cowan-1	Diffuse*	Diffuse	Compact
2	SE NFW	Diffuse	Diffuse	Diffuse
3	SE NTC	Diffuse	Diffuse	Diffuse
4	SA IPB	Diffuse	Diffuse	Diffuse
5	SA-Boyolali	Diffuse	Diffuse	Compact
6	SA-1	Diffuse	Diffuse	Compact
7	SA-2	Diffuse	Diffuse	Diffuse
8	SA-C1	Diffuse	Diffuse	Compact
9	SA-C5	Diffuse	Diffuse	Diffuse
10	SA-C6	Diffuse	Diffuse	Diffuse
11	SA-E1	Diffuse	Diffuse	Diffuse

*Colony formations

The presence of protein A on the surface of four respective *S. aureus* isolates was confirmed by the presence of *Spa* gene, which is responsible for the expression of protein A. The presence of *Spa* gene was further detected using PCR techniques (Table 2, Figure 2). The presence of the *Spa* gene can be detected in all isolates of *S. aureus* with compact colonies in rabbit serum-soft agar. On the contrary, the *Spa* gene could not be detected in all *S. aureus* with diffuse colonies in rabbit serum-soft agar (Figure 2).

These results suggested that there was a strong relationship between the colony formation of *S. aureus* and the presence of the *Spa* gene among *S. aureus* isolates. Serum-soft agar might be used for the simple and rapid method to detect the occurrence of protein A on the surface of *S. aureus* bacterial cells. The mechanism of the change of colony formation was not yet completely understood and should be further elucidated.

Table 2. The relationship between colony formation of *S. aureus* in rabbit SSA and the present of *Spa* gene for protein A

No	Colony Formation in Rabbit Serum-Soft Agar	Presence of Spa Gene
1	Compact:	
	SA Cowan 1	Yes
	SA Boyolali	Yes
	SA-1	Yes
	SA-C1	Yes
2	Diffuse:	
	Other isolates	No

Co-agglutination Anisakidae Rapid Test (Co-ART)

The protein A-bearing *S. aureus* isolate (SA-1), which had been coated with specific rabbit serum against WWE/L3/A, was used as the non-soluble matrix (NSM-SpA) and prepared for co-.agglutination Anisakidae-rapid test (Co-ART). The clear and distinct agglutination reactions can be observed in 3-5 seconds on object glass after one drop of Co-ART let to react with one drop of WWE/L3/A. No agglutination reactions were shown by the normal rabbit-coated NSM-SpA. (Figure 3). This finding indicated that the SA-IPB, protein A-positive *S. aureus*, can be used as non-soluble matrix components in preparing the Co-agglutination Anisakidae Rapid Test (Co-ART).

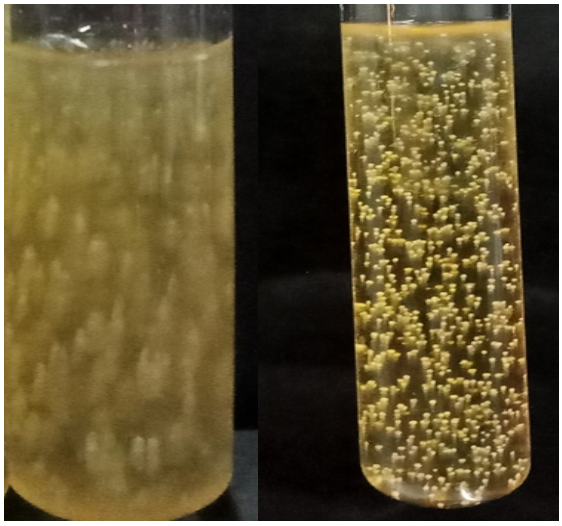


Figure 1. Diffuse growth of colony formation of SA-1 in chicken serum-soft agar (SSA+IgY, left) and change to compact colony formations in rabbit serum-soft agar (SSA+IgG, right)

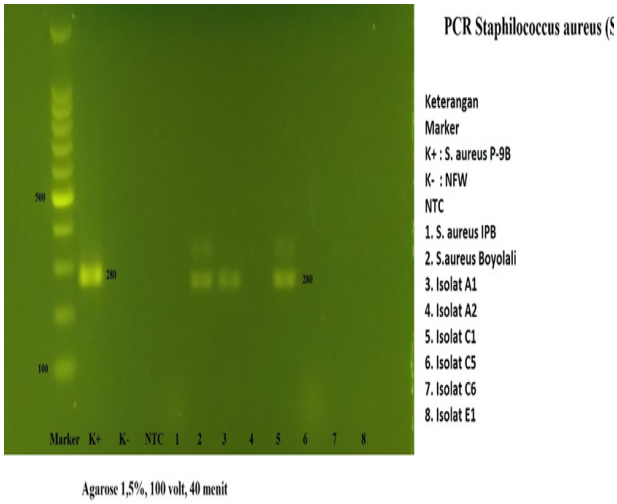
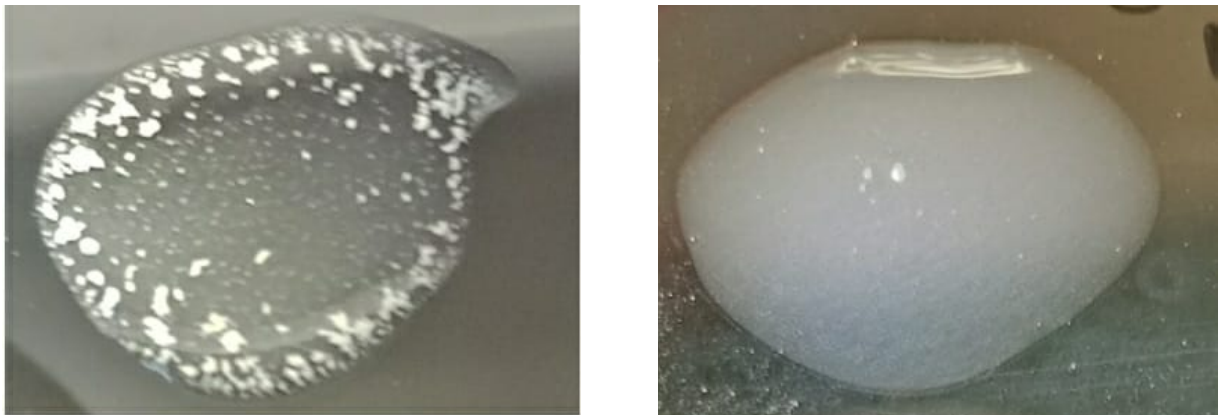


Figure 2. The PCR results of *Spa* gene (280 bp) which are expressed by *S. aureus* isolates with compact colony formation in rabbit serum-soft agar (SSA+IgG)



(A) **(B)**

Figure 3. Clear and distinct agglutination reactions were shown by the NSM-SpA matrix coated with the rabbit specific sera against WWE/A (anisakidae antigens) (A) and no agglutination reactions were expressed by NSM-SpA matrix coated with normal rabbit sera (B)

Staining of NSM-Spa matrix using methylene blue, safranin, or other dyes substances is a useful technique to enhance and contrast the agglutination reactions at the macroscopic level. Stains and dyes are also used to highlight the specimen at the microscopic level to study it at higher magnification for histopathological studies and diagnostic purposes (Ningrum *et al.*, 2017). The specific polyclonal L3 stage larvae of Anisakidae (L3/A) were used to enhance the recognition of antibodies to epitopes of L3/A antigens. The polyclonal antibodies' multi-epitope binding properties make them suitable for application in co-agglutination tests (Ascoli and Aggeler, 2018). The use of agglutination test using latex as a non-soluble matrix was used to detect the presence *Trichinella* antigen in meat (Gayda *et al*., 2016). The co-agglutination reaction is less time-consuming, requires less antigen and serum, and is less complicated compared to the immunodiffusion test, thus making it suitable for rapid screening.

CONCLUSION

The formation of compact colonies in rabbit SSA can distinguish the protein A-positive *S. aureus*, which was confirmed by *Spa* gene detection using PCR. The WWE/L3/A serum coated-protein A-bearing *S. aureus* could perform a definitive co-agglutination reaction. These findings provide a basic foundation for the development of WWE/L3/A antibody-coated protein A-based rapid serological test suitable for mass screening.

SUGGESTION

Further studies should be done due to the biological and immunological properties of antibody induced by antibody-anti-idiotype compared to the specific antibody of the anisakidae original antigen.

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