Identification of Protein A and Capsular Structures in *Staphylococcus aureus* Isolates from Milk of Cows with Subclinical Mastitis

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ABSTRAK

Putri RY, Windria S, Cahyadi AI. 2025. Identifikasi Struktur Protein A dan Capsular pada isolate *Staphylococcus aureus* susu sapi dengan mastitis subklinis. JITV 30(2): 82-91. DOI: http://dx.doi.org/10.14334/jitv.v30i2.3471.

Staphylococcus aureus merupakan salah satu penyebab masalah utama mastitis subklinis. Bakteri ini merupakan kelompok mikrobiota normal dan bisa menjadi bakteri patogen, karena kemampuannya menghasilkan toksin, invasif, dan ketahanan terhadap agen antimikroba. Proses patogenisitas *Staphylococcus aureus* salah satunya melibatkan faktor virulensi yang berada pada pemukaan bakteri dengan cara menghambat proses fagositosis, seperti protein A dan kapsul polisakarida. Identifikasi keberadaan protein A dan kapsul polisakarida dilakukan pada 28 isolat *Staphylococcus aureus* yang berasal dari bahan biologis tersimpan (BBT) dan diisolasi dari sapi penderita mastitis subklinis dengan hasil *California Mastitis Test* (CMT) ++ (positif 2). Keberadaan protein A dapat diidentifikasi menggunakan metode *serum soft agar* (SSA), sedangkan keberadaan kapsul polisakarida dapat diidentifikasikan menggunakan metode *salt aggregation test* (SAT). enelitian ini dilakukan untuk mendeteksi keberadaan faktor virulensi keduanya secara fenotipik. Hasil uji identifikasi protein A pada metode SSA menghasilkan 25 dari 28 isolat positif protein A dan kapsul polisakarida pada metode SAT menghasilkan seluruh isolat positif kapsul. Maka dari itu, kedua faktor virulensi tersebut dapat berkontribusi pada patogenisitas.

Kata Kunci: Kapsul, Polisakarida, Protein A, *Staphylococcus aureus*, Mastitis Subklinis, Virulensi

ABSTRACT

Putri RY, Windria S, Cahyadi AI. 2025. Identification of protein A and capsular structures in *Staphylococcus aureus* isolates from milk of cows with sublinical mastitis. JITV 30(2):82-91. DOI: http://dx.doi.org/10.14334/jitv.v30i2.3471.

Staphylococcus aureus is a primary etiological agent of subclinical bovine mastitis. While commonly part of the normal microbiota, it can transition into a pathogen through its capacity to produce toxins, invade host tissues, and resist antimicrobial agents. Its virulence is primarily linked to surface-expressed factors such as Protein A and polysaccharide capsules, which facilitate immune evasion by inhibiting phagocytosis. In this study, 28 *S. aureus* isolates were obtained from preserved biological materials (BBT) and assessed for the presence of Protein A and capsular structures. Protein A was identified using the serum soft agar (SSA) method, and polysaccharide capsules were detected via the salt aggregation test (SAT). Results showed that 25 of the 28 isolates expressed Protein A, while all exhibited capsule formation. These findings suggest that both virulence factors play a crucial role in the pathogenic potential of *S. aureus* in subclinical mastitis.

Key Words: Capsule, Polysaccharide, Protein A, *Staphylococcus aureus*, Subclinical Mastitis, Virulency

INTRODUCTION

Staphylococcus aureus is an infectious agent with high mortality and morbidity rates (Cheung et al. 2021). According to the study by Rao et al. (2023), livestock in India, especially in the Madurai district of Tamil Nadu, has recently experienced an increasing prevalence of *S. aureus* bacterial infections, with prevalence rates of approximately 42.63% in cattle and 28.57% in small ruminants. *Staphylococcus aureus* is a significant cause of subclinical mastitis (Windria et al. 2023; Ayuti et al. 2023). This statement is supported by the study conducted by Belay et al. (2022), which shows that bacterial pathogens isolated from mastitis cases are predominantly *Staphylococcus aureus* (42.6%), followed by *Streptococcus* spp. (26.2%) and other *Staphylococcus* species (26.2%). The incidence of *Staphylococcus aureus* infection has become a significant and critical issue in the dairy cattle industry (Pumipuntu et al. 2017).

Staphylococcus aureus infections are characterized by prolonged healing processes due to the persistence of the bacterium and its ability to evade the host immune system (Zhu et al. 2024). According to Windy et al. (2023), *S. aureus* exhibits complex pathogenicity within the host, facilitated by its virulence factors. The activity of these virulence factors is regulated by the accessory

gene regulator (*agr*) and the alternative transcriptional sigma factor SigB. The virulence factors of *S. aureus* include surface cell components and secreted proteins (Jenul & Horswill, 2019). Surface cell components, such as polysaccharide capsules and protein A, play a crucial role during the exponential growth phase by promoting bacterial adhesion and evading the host immune response (Wang & Muir 2016).

Protein A and polysaccharide capsules are the key virulence factors of *S. aureus*, playing crucial roles in evading the host immune response. Protein A inhibits opsonophagocytosis, acts as a B cell superantigen, and mediates inflammation. It interferes with the recognition of bacteria by phagocytic cells through its interaction with IgG (Fcγ immunoglobulin G), disrupting the opsonization process (Thammavongsa et al. 2015). Polysaccharide capsules further enhance immune evasion by obstructing the attachment of C3b to the bacterial surface, thereby inhibiting complement activation and phagocytosis. They also promote bacterial colonization during the adhesion phase, facilitate biofilm formation, and contribute to biofilm dispersion (Gao et al. 2024). The combined actions of protein A and polysaccharide capsules significantly enhance the pathogenicity of *S. aureus* by impeding phagocytosis (Husna 2018).

The identification of both virulence factors is necessary to determine their presence, as insufficient studies have been carried out on this topic. *S. aureus-producing* Protein A can be detected qualitatively using the serum soft agar (SSA) method (Evan et al. 2021), while polysaccharide capsules can be detected using the salt aggregation test (SAT) method (Khusnan & Kusmanto 2019). Both methods aim to determine the presence of virulence factors on the bacterial surface that contribute to the pathogenicity of subclinical mastitis. This study aims to identify *S. aureus* bacteria that produce the virulence factors Protein A and polysaccharide capsules.

MATERIALS AND METHODS

Sample

The samples used in this study are biological materials, specifically 28 preserved isolates of *Staphylococcus aureus*. The samples were derived from the milk of dairy cows diagnosed with mastitis subclinis, confirmed by a positive result (2++) on the California Mastitis Test (CMT). The isolates used as samples are preserved biological materials in the Microbiology Laboratory of the Faculty of Medicine, Universitas Padjadjaran, preserved in 30% glycerol medium at -20°C.

Re-identification of preserved isolates

Staphylococcus aureus was cultured on a blood agar plate (BAP) to observe colonies with a round shape and white to golden color. The procedure for culturing on BAP involved sampling with a loop, followed by incubation for 24 hours at 37°C. A hemolysis zone will form around the bacterial colonies, as the pathogenic bacteria produce a hemolysin toxin that causes lysis of the cytoplasmic membrane in the blood (Windria et al. 2023).

The Gram staining procedure involves preparing a smear of the sample on a glass slide, inoculated from BAP media using a loop, and then heating it with a Bunsen flame. Then, the smear was stained with crystal violet, allowed to stand for 2 minutes, and rinsed with water. Next, Lugol's iodine solution is applied to the smear, left for 30 seconds, and rinsed with water. The smear is then dipped in 96% alcohol to decolorize the stain, followed by immediate rinsing with water. Finally, the smear is counterstained with safranin, left for 2 minutes, and rinsed with water. A drop of immersion oil is applied to the dried smear, and the sample is observed under a microscope at 100x Magnification. *Staphylococcus aureus* is a Gram-positive bacterium characterized by its purple color (forming grape-like clusters) and coccal shape (Hayati et al. 2019).

Bacterial isolation on mannitol salt agar (MSA) was performed by taking a single loop of the sample and inoculating it onto the mannitol medium, followed by incubation at 37°C for 24 hours. The color change of *Staphylococcus aureus* from red to yellow is attributed to its ability to ferment mannitol (Thakur et al. 2017; Darmawi et al., 2019).

The coagulase test is performed to detect the presence of the coagulase enzyme. The test was conducted by inoculating bacteria into 1 mL of Nutrient Broth using a loop, followed by incubation at 37°C for 24 hours. Subsequently, rabbit plasma containing citrate was added to the Nutrient Broth with the bacteria, and the mixture was incubated again for 24 hours. The presence of clots at the bottom of the Eppendorf tube indicates a positive result (Hayati et al. 2019).

The catalase test is a method used to differentiate between the genera of *Staphylococcus* and *Streptococcus*. The catalase test was performed by taking a bacterial colony from MSA media using a loop and placing it on a glass slide. Then, 1–2 drops of H_2O_2 were added to the slide and mixed with the bacteria. The presence of gas bubbles (O_2) indicates a positive result (Yanto et al. 2021).

The DNase test is used to identify pathogenic *Staphylococcus* species, such as *Staphylococcus aureus*. The procedure begins with inoculating the culture onto a DNase agar plate and incubating at 37°C. After incubation, the agar plate is flooded with HCl and left for

approximately 5 minutes. The formation of a clear zone around the colonies indicates a positive result (Karimela et al. 2018).

Identification of Protein A Presence by Serum Soft Agar (SSA) Method

The test procedure involves inoculating bacteria into Todd Hewitt Broth (THB) medium and incubating it at 37°C for 18–24 hours. Soft agar (SA) preparation consists of 0.15% agar base in 10 mL of Brain Heart Infusion (BHI) medium. The soft agar (SA) is heated until the liquid becomes homogeneous and then cooled to 37–40°C. Bacteria from the THB medium are collected using a loop, inoculated into 10 mL of physiological NaCl, and homogenized until the bacterial cells are evenly mixed. Next, 100 µL of rabbit serum is added to the prepared soft agar (SA). A bacterial suspension from the THB medium is then transferred using a loop into the serum soft agar (SSA) mixture, homogenized with a vortex, and incubated at 37°C for 18–24 hours. The formation of compact colonies in the tube indicates a positive result (Djannatun et al. 2016).

Identification of Polysaccharide Capsule Presence by Salt Aggregation Test (SAT)

The test begins by inoculating bacteria into 5 mL of Brain Heart Infusion (BHI) medium and incubating at 37°C for 24 hours. The culture is vortexed and transferred into a tube, followed by centrifugation for 5 minutes at 5000 rpm. The supernatant is discarded, and the resulting pellet is washed three times with Phosphate Buffer Saline (PBS). The bacterial suspension in BHI is adjusted to a concentration of 10^8 cells/mL by matching it to a $BaSO_4$ standard. Once the desired concentration is achieved, 50 µL of the bacterial suspension is mixed with 50 µL of ammonium sulfate at concentrations of 1.2 M, 1.6 M, 2 M, 2.4 M, and 3.2 M on a glass slide, then homogenized using a sterile toothpick. The absence of aggregation on the glass slide will identify *Staphylococcus aureus* with polysaccharide capsules (Khusnan & Kusmanto 2019).

Data analysis

The presence of virulence factors, such as Protein A and polysaccharide capsules, in *Staphylococcus aureus* bacteria is qualitatively detected using the serum soft agar (SSA) method and the salt aggregation test (SAT), which are performed after re-identification of preserved bacterial isolates. The data obtained from these methods show phenotypic changes in the media used. The research results are presented in a table that contains the positive and negative outcomes of the tests, along with an interpretation of their characteristics. Data analysis will be conducted descriptively to describe the results.

Ethical Approval

This study received ethical approval from the Research Ethics Committee of the Faculty of Medicine (Approval No. 903/UN6.KEP/EC/2024).

RESULT AND DISCUSSION

Re-identification of preserved isolates

Staphylococcus aureus cultured on a blood agar plate (BAP) medium showed that 24 isolates exhibited β-hemolysin activity, while four isolates exhibited α-hemolysin activity (Table 1). The characteristics of hemolysis types can be observed based on the zones formed (Sodiq et al. 2019). β-hemolysis produces a clear zone, referred to as complete hemolysis (Figure 1A), while α-hemolysis forms a dark zone with partial clearing, referred to as partial hemolysis (Figure 1B). *Staphylococcus aureus* can exhibit four types of hemolysins: β, α, γ, and δ (Artursson et al. 2016; Turista & Puspitasari 2019; Divyakolu et al 2019; Wang et al. 2020). According to Turista & Puspitasari (2019), β and α-hemolysins are the most critical determinants in the pathogenic process of *Staphylococcus aureus*. Hemolysins exert cytotoxic effects, inducing cell lysis (Pakshir et al., 2017). Specifically, these bacteria contribute to mastitis by exacerbating necrosis in mammary gland tissues (Pérez et al. 2020; Campos et al. 2022). According to Abril et al. (2020), hemolysin in *S. aureus* plays a dominant role in the development of subclinical mastitis in dairy cows. Hemolysin is responsible for inflammation and injuries to the mammary gland epithelium, which increases tissue loss and leads to the manifestation of mastitis (Demontier et al. 2020).

The results of testing 28 isolates on mannitol salt agar (MSA) showed that 25 isolates produced positive results, while 3 isolates were negative (Table 1). Similar findings were reported in a study by Santos et al. (2015), which noted that 9 out of 59 *S. aureus* isolates yielded negative MSA results. Positive results are characterized by a color change from red to yellow (Figure 1B (a)), while negative results retain the red color (Figure 1B (b)). The color change occurs due to the fermentation of mannitol into organic acids, which causes the pH indicator phenol red in the medium to change. *Staphylococcus aureus* possesses an enzyme critical for mannitol metabolism, namely Mannitol-1-phosphate dehydrogenase (M1PDH) (Nguyen et al. 2019).

In this study, the results of Gram staining indicated that all isolates exhibited the characteristic traits of *S.*

Table 1. Re-identification results of 28 isolates preserved from the milk of dairy cows causing subclinical mastitis with CMT results 2++ (positive 2)

No.	Hemolysis Type	Gram staining	MSA	Catalase	Coagulase	DNAse	Interpretation
1.	β	coccus +	+	+	+	+	*S. aureus*
2.	α	coccus +	+	+	+	+	*S. aureus*
3.	β	coccus +	+	+	+	+	*S. aureus*
4.	β	coccus +	+	+	+	+	*S. aureus*
5.	β	coccus +	+	+	+	+	*S. aureus*
6.	β	coccus +	+	+	+	+	*S. aureus*
7.	β	coccus +	+	+	+	+	*S. aureus*
8.	β	coccus +	+	+	+	+	*S. aureus*
9.	β	coccus +	-	+	+	+	*S. aureus*
10.	β	coccus +	+	+	+	+	*S. aureus*
11.	β	coccus +	+	+	+	+	*S. aureus*
12.	β	coccus +	+	+	+	+	*S. aureus*
13.	α	coccus +	+	+	+	+	*S. aureus*
14.	β	coccus +	-	+	+	+	*S. aureus*
15.	β	coccus +	-	+	+	+	*S. aureus*
16.	β	coccus +	+	+	+	+	*S. aureus*
17.	β	coccus +	+	+	+	-	*S. aureus*
18.	α	coccus +	+	+	+	+	*S. aureus*
19.	β	coccus +	+	+	+	-	*S. aureus*
20.	α	coccus +	+	+	+	+	*S. aureus*
21.	β	coccus +	+	+	+	+	*S. aureus*
22.	β	coccus +	+	+	+	+	*S. aureus*
23.	β	coccus +	+	+	+	+	*S. aureus*
24.	β	coccus +	+	+	+	+	*S. aureus*
25.	β	coccus +	+	+	+	+	*S. aureus*
26.	β	coccus +	+	+	+	+	*S. aureus*
27.	β	coccus +	+	+	+	+	*S. aureus*
28.	β	coccus +	+	+	+	+	*S. aureus*
Total	α = 4 β = 24	28	+ = 25 - = 3	28	28	+ = 26 - = 2	

α= Alpha, β= Beta,γ= Gamma, Coccus += Gram Positive, += Positive, - = Negative

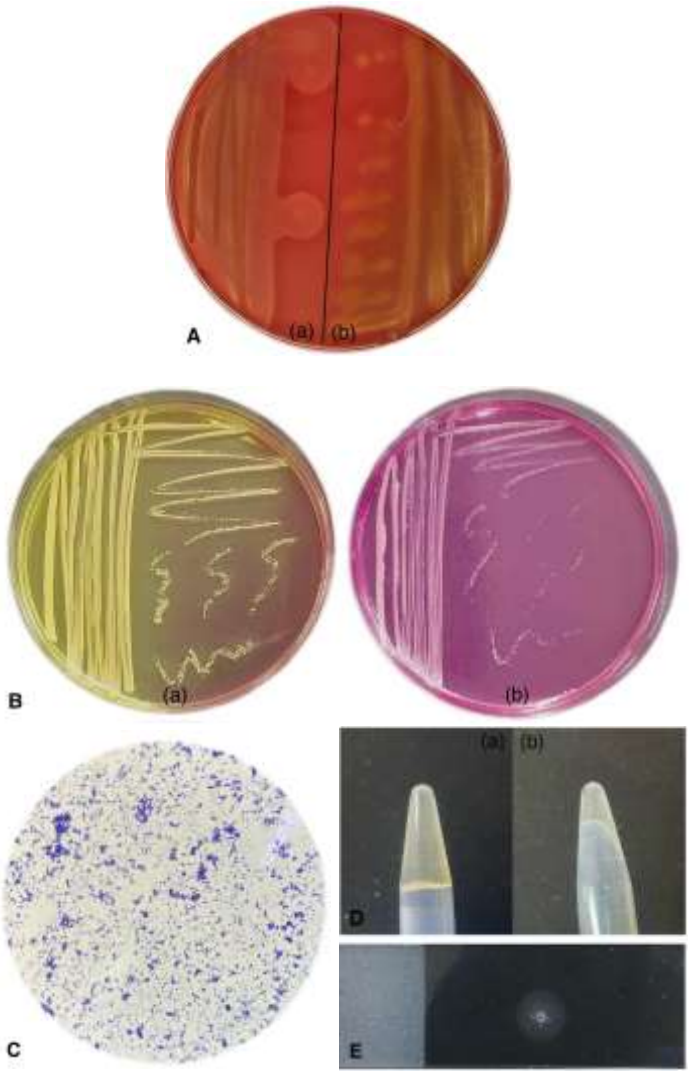

Figure 1. Identification Results of Staphylococcus aureus. (A) BAP Showing Hemolysis Zones (a) β-hemolysis (b) α-hemolysis, (B) MSA Showing (a) Positive mannitol fermentation (b) Negative mannitol fermentation, (C) Gram staining Results at 100x Magnification, (D) Coagulase Test (a) Clump formation (b) No clump formation, (E) Catalase Test: Presence of gas bubbles, (F) DNase Test (a) Presence of a clear zone around the colony (b) No clear zone formation. (Personal Documentation)

aureus, including a coccoid shape, a clustered arrangement, and a purple coloration (Figure 1C). These findings are consistent with those reported by Darmawi et al. (2019), which described similar traits and categorized *S. aureus* as a Gram-positive bacterium. The bacterial cell wall consists of layers of peptidoglycan, lipoteichoic acid (LTA), wall teichoic acids (WTA), and surface proteins (Wang et al. 2022). The ability to retain the stain is attributed to the thick peptidoglycan layer, which dehydrates upon alcohol exposure, causing the cell wall pores to close and trap the crystal violet dye within the cell (Yanto et al. 2021). Gram-positive bacteria such as *Staphylococcus aureus* tend to elicit a slower immune and inflammatory response. However, in mastitis cases, they are more likely to cause chronic infections compared to Gram-negative bacteria (Günther et al. 2017).

Staphylococcus aureus has a clotting factor called protein coagulase (*coa*). The results of this study showed that 28 samples produced the coagulase enzyme, resulting in the formation of a clot at the bottom of the Eppendorf tube (Figure 1D(a)). The process of interaction between the host cell plasma and coagulase activates thrombin to form fibrin, a clotting factor (Hayati et al. 2019). The clotting factor formed provides an opportunity for *S. aureus* bacteria to evade the immune response, as the immune system does not detect the fibrin formed, thereby preventing the phagocytosis process (Crosby et al. 2016). Staphylococcus aureus, which causes mastitis, produces coagulase as its virulence factor (Suwito et al. 2024).

The catalase test results showed that all isolates were positive for catalase enzyme production, as indicated by the formation of gas bubbles (Figure 1E). These findings

align with studies by Azis et al. (2022), which demonstrated that *Staphylococcus aureus* possessing the catalase enzyme produces gas bubbles in the test. This enzyme breaks down hydrogen peroxide (H_2O_2) into oxygen (O_2) and water, providing bacterial defense against reactive oxygen species (ROS) (Yanto et al. 2021). Catalase is one of the factors that enable bacteria to survive during immune responses (Karimela et al. 2019).

Staphylococcus aureus produces an extracellular enzyme known as deoxyribonuclease (DNase) (Subathra et al. 2016). This study yielded positive DNase test results in 25 isolates and negative results in 3 isolates. Positive results were indicated by the formation of a clear zone around the colonies, while negative results showed no clear zone (Figure 1F). These findings align with the study conducted by Pumipuntu et al. (2017), which reported 26 positive samples and 2 negative samples. Negative results in this test may indicate the occurrence of false negatives (Windria et al. 2023), consistent with the findings of Pumipuntu et al. (2017), who also observed false negatives in DNase tests.

Mastitis is an inflammation of the mammary gland, characterized by its complexity, particularly in terms of origin, severity, and outcomes (Thompson-Crispi et al., 2014; Saleem et al., 2024). According to Haxhiaj et al. (2022) and Huma et al. (2022), *Staphylococcus aureus* is a common pathogenic bacterium responsible for mastitis.

Positive results occur because Protein A interacts by forming bonds with the Fc receptor on IgG, which is found in various mammalian species (Lestari & Salasia 2017). The compact colonies formed on SSA can provide insight into the persistence and chronicity of this infection in subclinical mastitis (Ningrum et al. 2016). Negative results have two possible explanations: either the bacteria lack Protein A, or Protein A is present but concealed by the bacterial capsule (Lestari & Salasia 2017).

Factors affecting the results of the SSA test include the dilution process using 9% NaCl and the homogenization process during vortexing, as the SSA method is sensitive to the homogenization process. The release activity of Protein A on the surface of

In this study, the identification of polysaccharide capsules using the salt aggregation test (SAT) showed that all 28 isolates were positive for polysaccharide capsules. Positive results were indicated by the absence of aggregation and hydrophilic properties at specific concentrations of ammonium sulfate. A study by

S. aureus possesses various virulence factors that contribute to subclinical mastitis infection and support pathogenesis in response to the host's immune system (Pérez et al. 2022)

Identification of the presence of protein A and polysaccharide capsules

Identification of Protein A using the serum soft agar (SSA) method in 28 phenotypically cultured isolates resulted in 25 positive isolates and 3 negative isolates for Protein A. Positive results were indicated by compact colonies. In contrast, negative results showed diffuse colonies (Figure 6). In the study by Djannatun et al. (2016), 7 out of 15 *Staphylococcus aureus* isolates showed positive results, characterized by a change in colony morphology from diffuse to compact following interaction with rabbit serum on SSA.

Staphylococcus aureus is influenced by the presence of sortase A (srtA). If sortase A is absent, the attachment of surface proteins, including Protein A, will be disrupted (Ningrum et al. 2016).

The virulence factor Protein A is classified as a structural surface component that contributes to the progression of mastitis (initiating infection in the mammary gland) caused by *Staphylococcus aureus* (Tegegne et al. 2021). The interaction between Protein A and the Fc region of IgG interferes with the immune response by inhibiting opsonization and phagocytosis. This response creates favorable conditions for *Staphylococcus aureus* to proliferate and cause infection (Lestari & Salasia, 2017). Protein A is responsible for intramammary infection (Demontier et al. 2020). Khusnan et al. (2016) found that *Staphylococcus aureus* isolates were 85.7% hydrophilic and 14.3% hydrophobic.

The use of ammonium sulfate at specific concentrations serves as an indicator for determining the degree of hydrophobicity, with its activity determined by

Table 2. Identification results of the presence of protein A and polysaccharide capsules in *Staphylococcus aureus* bacteria from the milk of bovine subclinical mastitis

	Protein A in serum soft agar (SSA)	Polysaccharide Capsules in salt aggregation test (SAT)
Positive	25	28
Negative	3	0
Total	28	

Protein A-positive isolates exhibit compact colony morphology, while polysaccharide capsule-positive isolates are hydrophilic, with aggregation occurring at concentrations >2 M

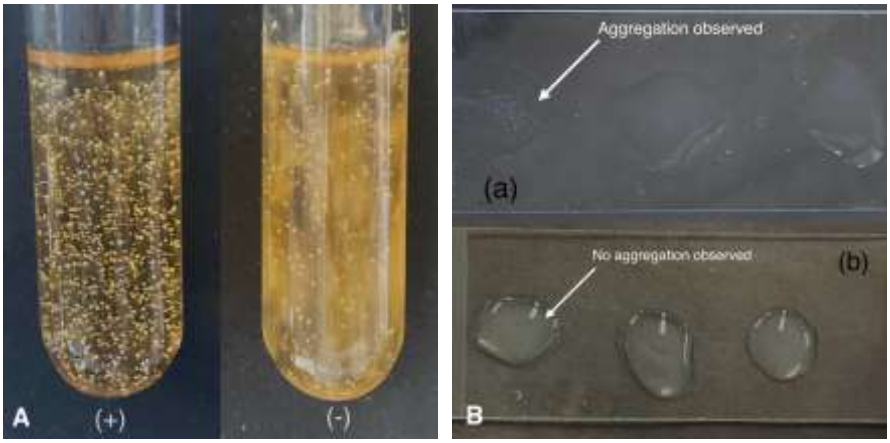

Figure 2. Identification results on *Staphylococcus aureus* (A) The presence of protein A (+) Positive for protein A with compact colonies (-) Negative for protein A with diffuse colonies, (B) The presence of Polysaccharide Capsule (a) Aggregation was observed (b) No aggregation was observed. (Personal Documentation)

water attraction and protein precipitation (Prasiddhanti & Wahyuni 2015). Farid et al. (2021) further explained that the degree of hydrophobicity is categorized into two types: hydrophilic, if aggregation occurs at ammonium sulfate concentrations greater than 2.0 M, and hydrophobic, if aggregation occurs at ammonium sulfate concentrations between 1 and 2 M. All isolates showed positive results for polysaccharide capsules, as none formed aggregates during observation on a glass slide. Three isolates formed aggregates only at a concentration of 3.2 M (Figure 2B(a)), while no aggregation was observed at lower concentrations. According to the explained theory, if aggregation occurs at concentrations greater than 2 M, the isolate is classified as hydrophilic, indicating that the *Staphylococcus aureus* bacteria in the isolates from this study possess capsules on their surface (Khusnan & Kusmanto 2019).

There is a correlation between the hydrophobicity properties and virulence of *S. aureus* bacteria, where hydrophilic bacteria are more pathogenic compared to hydrophobic ones (Khusnan & Kusmanto 2019). The polysaccharide capsule of *S. aureus* is responsible for the adhesin process, or the attachment of the bacterium to the host, particularly to epithelial cells (Khusnan & Kusmanto 2019). According to Salimena et al. (2016), the polysaccharide capsule is responsible for chronic and persistent infections in bovine mastitis. The polysaccharide capsule facilitates the initial adhesion/attachment process of biofilm formation (Vidlund et al. 2021). This biofilm activity causes *S. aureus* to further spread the infection process in mastitis cases.

CONCLUSION

The presence of virulence factors, including protein A and polysaccharide capsules, was identified phenotypically in 28 preserved isolates of *Staphylococcus aureus* bacteria. *Staphylococcus aureus* in this study has a protein A virulence factor (25 isolates out of 28 isolates) and a polysaccharide capsule (28 isolates). *Staphylococcus aureus* bacteria, identified by the virulence factor protein A and polysaccharide capsules on their surface, are more pathogenic. The resulting impact is the expression of pathogenicity. Both virulence factors contribute to mastitis infection in cows.

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