Biofilm Profile of Coagulase Negative *Staphylococci* Bacteria from Milk Isolate of Dairy Cows with Subclinical Mastitis

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ABSTRAK

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Staphylococcus sp. merupakan bakteri patogen penyebab mastitis subklinis. Bakteri ini terbagi menjadi kelompok bakteri *coagulase negative Staphylococci* (CoNS) dan kelompok bakteri *coagulase positive Staphylococci* (CoPS). Bakteri CoNS merupakan kelompok flora normal pada kulit manusia dan hewan, walaupun demikian beberapa penelitian telah membuktikan bahwa bakteri CoNS merupakan mikroorganisme yang paling banyak diisolasi dari susu sapi perah penderita mastitis subklinis. Kemampuan untuk membentuk biofilm merupakan faktor virulensi yang penting bagi bakteri CoNS. Deteksi pembentukan biofilm dilakukan pada 54 sampel bakteri CoNS berupa Bahan Biologi Tersimpan (BBT), yang diisolasi dari susu sapi perah penderita mastitis subklinis dengan hasil uji *California mastitis test* (CMT) positif 2 (++). Deteksi pembentukan biofilm dilakukan secara kualitatif dengan metode *Congo red agar* (CRA) dan *test tube* (TT). Hasil konfirmasi secara fenotipe, menunjukan bahwa 54 isolat (100%) merupakan bakteri CoNS. Hasil deteksi pembentukan biofilm menunjukkan hasil bahwa 51 dari 54 isolat (94,44%) positif membentuk biofilm. Sehingga, dapat disimpulkan bahwa bakteri CoNS memiliki kemampuan membentuk biofilm sebagai salah satu bentuk perlindungan diri dan faktor virulensi.

Kata Kunci: *Coagulase-negative Staphylococci*, Faktor virulensi, Mastisis Subklinis

ABSTRACT

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Staphylococcus sp. is a pathogenic bacteria that causes subclinical mastitis. These bacteria are divided into coagulase-negative *Staphylococci* (CoNS) and coagulase-positive Staphylococci (CoPS). CoNS bacteria are a group of normal flora on human and animal skin. However, several studies have proven that CoNS bacteria are the most commonly isolated microorganisms from the milk of dairy cows with subclinical mastitis. The ability to form biofilms is an important virulence factor for CoNS bacteria. Detection of biofilm formation was carried out on 54 samples of CoNS bacteria in the form of Stored Biological Material (BBT), which were isolated from the milk of dairy cows with subclinical mastitis with positive California mastitis test (CMT) 2 (++). Detection of biofilm formation was performed qualitatively by Congo red agar (CRA) and test tube (TT) methods. Phenotypic confirmation results showed that 54 isolates (100%) were CoNS bacteria. Biofilm formation detection results showed that 51 out of 54 isolates (94.44%) were positive for biofilm formation. Thus, it can be concluded that CoNS bacteria have the ability to form biofilms as a form of self-protection and virulence factor.

Key Words: Biofilm, Coagulase-negative Staphylococci., Subclinical Mastitis, Virulence Factor

INTRODUCTION

Mastitis is one of the causes of decreased milk production in dairy cows (Panjuni et al. 2021). Clinical manifestations of mastitis are divided into clinical mastitis and subclinical mastitis (Suwito et al. 2021). Subclinical mastitis in Indonesia reaches 97-98%, while clinical mastitis only reaches 2-3% (Nisa et al. 2019). This physical clinical symptom does not appear, causing dairy cows with subclinical mastitis to become a reservoir that infects other dairy cows (Pribadi et al. 2020).

Staphylococcus sp. is the most common pathogenic bacteria found in cases of subclinical mastitis (Suwito et al. 2021). These bacteria are divided into two groups based on their ability to produce coagulase enzymes: coagulase-negative *staphylococci* (CoNS) and coagulase-positive *staphylococci* (CoPS). Identification of pathogenic microorganisms for the incidence of subclinical mastitis in dairy cattle is still concentrated in CoPS bacteria, such as Staphylococcus aureus, rather than CoNS bacteria, such as *Staphylococcus epidermidis* (Windria et al. 2016). CoNS bacteria are normal flora of the skin. This emerging bacterial pathogen causes

mastitis in dairy cows in countries such as Germany, Africa, Iran, and Egypt (Hosseinzadeh and Dastmalchi Saei 2014).

Mastitis infection caused by CoNS bacteria is subclinical but persistent (Cheng and Han 2020). CoNS bacteria can produce biofilms, an important factor in their pathogenicity (Goetz et al. 2017). The presence of biofilms causes bacteria to become more persistent on milking tools and hands, which are portals of entry for bacterial infections (El-Jakee et al. 2013). The formation of biofilms also causes an increase in the resistance and adaptation of CoNS bacteria to the environment in the animals' udders, so it becomes a problem in the treatment and prevention of subclinical mastitis in dairy cows (De Buck et al. 2021).

Biofilm is a collection of multicellular microorganisms in a matrix of extracellular polymeric substances. The existence of biofilms causes bacteria to be more able to attach to the surfaces of biotic and abiotic objects, as well as thickening the protective layer of bacteria (França et al. 2021). Bacteria in the biofilm can carry out synergism or positive interactions between bacteria, thus causing an increase in the pathogenicity of these bacteria (Espiritu and Villanueva 2022). This condition can lead to failure to treat subclinical mastitis in dairy cows, increasing antibiotic resistance and threatening animals and humans.

The ability to form bacterial biofilms can be detected quantitatively or qualitatively. The Congo red agar (CRA) method and the test tube method (TT) are qualitative test methods that can be used to detect the ability to form biofilms of CoNS bacteria. The CRA and TT methods have the advantage of fast processing time and easy result analysis because they are only based on visual changes in the media (Furtuna et al. 2018).

Information and research on CoNS bacteria related to the incidence of subclinical mastitis in dairy cattle in Indonesia is still very limited. CoNS bacteria have virulence factors that cause subclinical mastitis in dairy cows, namely their ability to form biofilms that need to be studied. This study was conducted as a test to detect the ability of biofilm formation as a virulence factor from CoNS bacteria from milk isolates of dairy cows with subclinical mastitis.

MATERIALS AND METHODS

Sample

The research sample used was stored biological material of 54 samples isolate bacteria, which had been isolated from milk samples of dairy cows with subclinical mastitis with positive California mastitis test (CMT) results of 2 (++). The bacterial isolates used as samples were stored in STGG media (skim milk, tryptone, glucose, glycerol) and 30% glycerol.

Method

Confirmation of Stored Isolates by Phenotype

A bacterial culture was carried out on blood agar plate (BAP) media to obtain CoNS bacterial colonies. This procedure was carried out by taking 1 dose of inoculum from the sample and then inoculating it on BAP media. The agar plate was then incubated at 37°C for 24 hours. Three types of hemolysis zones can form on BAP media, namely β-hemolysis (clear zone), which is formed due to complete lysis of erythrocytes, α-hemolysis (brownish green zone), which is formed due to bacteria reducing erythrocyte hemoglobin to methemoglobin, and γ-hemolysis. Or non-hemolytic (Almwafy et al. 2020).

Gram staining was performed to see the morphology of the bacteria. The smear preparation for Gram staining was made on an object glass by taking 1 dose of inoculum from BAP media and fixing it. The smear preparation was dripped with crystal violet for 2 minutes and then rinsed with running water. The smear preparation is then dripped with Lugol's solution, left for 30 seconds, and rinsed with running water. The next step is to fade the dye on the smear using 96% alcohol, then rinse it with running water. The smear preparation was stained again by dripping safranin dye, left for 2 minutes, rinsed with running water, and dried. Observations were made under a microscope using an objective lens magnification of 100x using immersion. CoNS bacteria are Gram-positive, non-motile, non-spore-forming cocci that form tetrads or in pairs but can also occur individually, in irregular groups (forming grapes), or in short chains composed of three or four cells (Becker et al. 2014).

Bacterial isolation was carried out on the differential media of mannitol salt agar (MSA). The procedure was carried out by taking 1 dose of inoculum from the results of bacterial culture on nutrient agar (NA) media, then inoculating it into MSA media and then incubating it for 24 hours at 37°C. CoNS bacteria cannot ferment mannitol, so the bacteria will grow without causing a color change (from red to yellow) in the media (Ryman et al. 2021).

The catalase test was performed to differentiate the genera *Staphylococcus* sp. and *Streptococcus* sp. The test will be carried out by dripping liquid hydrogen peroxide (H_2O_2) on an object glass, and mixed it with the bacteria from the BAP media. A positive result is indicated by the presence of gas bubbles (O_2) produced by *Staphylococcus* sp. because it breaks down hydrogen peroxide (Hayati et al. 2019).

A coagulase test was performed to detect the presence of coagulase enzymes. This test procedure was carried out by taking bacterial isolates with loops from BAP media, putting them into 1 ml of nutrient broth in a tube, then incubating them for 24 hours at 37°C. 1 ml of rabbit blood plasma is put into the media using a syringe and then incubated for 24 hours. The results of the CoNS bacterial test did not show plasma clots that congealed like a gel in the tube (Becker et al. 2014).

Biofilm detection with the Congo Red Agar (CRA) method

CRA media was prepared with 37 grams/liter of brain heart infusion broth (BHIB), 50 grams/liter of sucrose, 10 grams/liter of agar base, and 0.8 grams/liter of Congo red indicator. Congo red indicator was prepared as a concentrated solution and then autoclaved at 121°C for 15 minutes. Add Congo red indicator into brain heart infusion agar with sucrose stored in a petri dish and autoclaved at 55°C. CRA plate was inoculated with CoNS and then incubated at 37°C for 24 – 72 hours. A positive result in the CRA test method is indicated by the formation of black-pigmented colonies with a rough consistency and black zones around the colonies. In contrast, a negative result is indicated by the formation of reddish-pigmented colonies with a smooth consistency (Furtuna et al. 2018).

Biofilm detection with the Test Tube (TT) method

The bacteria were inoculated into a tube containing 10 ml of Tryptone Soya Broth and 1% glucose, then incubated for 24 – 30 hours at 37°C. The tube is washed with phosphate-buffered saline (pH 7.3) and dried. The dry tube was then fixed with Bunsen and stained with 0.1% crystal violet for 10 minutes. Wash the tube with distilled water, then dry it by storing the tube upside down at room temperature. Observations were made by observing whether or not a blue layer was formed on the bottom and wall of the tube. If a ring was found at the boundary of the liquid, it was ignored because it is not an indicator of biofilm formation (Benachinmardi et al. 2017). Interpretation of the results in this study was not carried out by scoring or using a nephelometer, only visual observations were made, and the results were divided into positive and negative. The formation of a blue coating on the walls and bottom of the tube indicates that the bacteria form biofilms.

Data analysis

The ability to form biofilms of CoNS bacteria was detected using qualitative methods, CRA and TT, after confirming stored isolates to determine the test sample. The data obtained is the change in color of the test medium (CRA or TSB) after inoculating the bacterial sample. The results of the study were analyzed descriptively, then presented in a tabular form containing the positive and negative results of the tests, conclusions, and the percentage of CoNS bacteria from milk isolates of dairy cows with subclinical mastitis which can form biofilms. CoNS bacteria are considered to have the ability to form biofilms if the results of one or both tests are positive. The data obtained was then calculated using Microsoft Excel and the Cohen's Kappa value was determined for its reliability value.

RESULTS AND DISCUSSION

Phenotypic test

In this study, the microscopic images obtained from isolates of CoNS bacteria were coccus-shaped bacterial colonies grouped irregularly, non-motile, and purple (Table 1). This result is similar to that of Ahmadunissah et al. (2021), which stated that *Staphylococcus* sp. belongs to the group of Gram-positive bacteria. The grouping of bacterial types is based on the outermost layer of the bacterial wall, namely lipopolysaccharide, which only Gram-negative bacteria have, as well as the thickness of the peptidoglycan layer (Panawala 2017). The cell wall of Gram-positive bacteria has a thicker peptidoglycan layer than Gram-negative bacteria. It contains teichoic acid (TA), an anionic polymer of polyol phosphate repeating units (Kho and Meredith 2018). Bacterial colonies turn purple because alcohol causes the cell walls to dehydrate and their pores to shrink, causing the crystal violet and lugol complexes to bind to the peptidoglycan layer on the bacterial cell wall (Thairu et al. 2014).

This study showed that samples of the CoNS bacterial isolates reacted positively to produce the catalase enzyme, which was indicated by the formation of bubbles (Table 1). These results are similar to research by Kartini (2020), which stated that Staphylococcus sp. can produce catalase enzymes so that it can break down hydrogen peroxide (H_2O_2) into water (H_2O) and gas (O_2). Catalase is an antioxidant enzyme that plays a role in the defense of bacteria against oxidative stress by catalyzing the decomposition of H_2O_2(Yuan et al. 2021). The catalase enzyme in milk can come from bacteria or mammary glands (Eslami et al., 2015). Increased activation of the catalase enzyme will go hand in hand with an increase in the number of somatic cells in milk, which indicates mastitis (Zeinhom et al. 2013).

The coagulase test results in this study showed that 54 isolates (100%) had a negative reaction (Table 1). The coagulase enzyme protects bacteria from host immune cells by binding and activating prothrombin, forming a pseudo capsule of fibrin as a protective barrier for

Table 1. Phenotypic test results of bacterial isolates from milk of dairy cattle suffering from subclinical mastitis

Test	Results	Sample (n)	Percentages (%)
	β	33	61.11
Hemolysis Type	α	0	0
	γ	21	38,89
Gram Staining	Coccus +	54	100
	Coccus -	0	0
Mannitol Salt Agar	+	46	85.19
	-	8	14.81
Catalase	+	54	100
	-	0	0
Coagulase	+	0	0
	-	54	100
Total		54	100

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

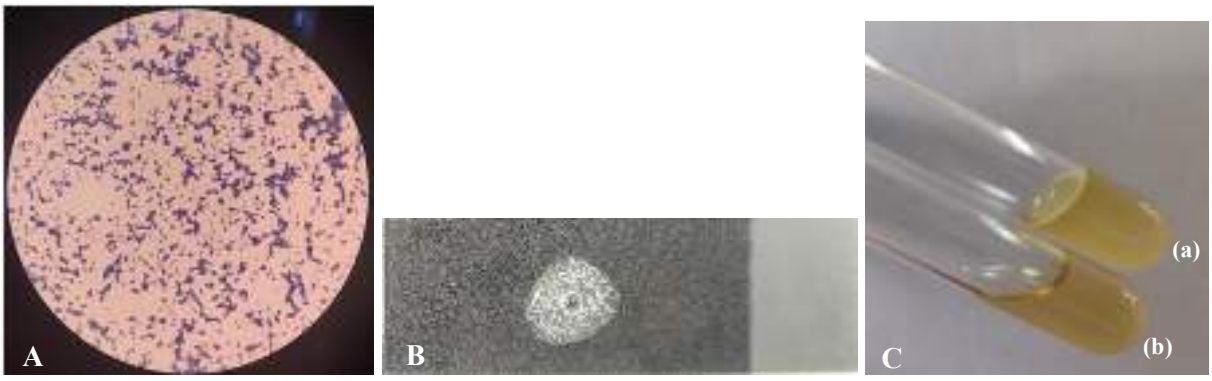

Figure 1. Phenotypic test results for coagulase negative Staphylococci (CoNS) bacteria **(A)** Gram Staining Results with 100x magnification, (B) Catalase Test Results, (C) Coagulase Test Results (a) Positive, (b) Negative (Personal Documentation)

bacteria (Tam & Torres 2019). CoNS bacteria do not produce this enzyme, so they are considered less pathogenic than CoPS bacteria (Roman et al. 2023). The results of this study showed that 21 of 54 samples (38.89%) were γ-hemolytic (Figure 2A), while 33 of 54 samples (61.11%) were β-hemolytic (figure 4b). 18 out of 21 isolates (85.71%) that were γ-hemolytic and 28 out of 33 isolates (84.85%) that were β-hemolytic could ferment mannitol (figure 5a). Three isolates that were γ-hemolytic (14.29%) and 5 isolates (15.15%) that were β-hemolytic could not ferment mannitol (Figure 3B).

This study's results differ from Kim et al. (2019) statement that CoNS bacteria are non-hemolytic, so they do not form a hemolysis zone on BAP. Research Organji et al. (2018) stated that the CoNS bacterial species could not ferment mannitol, contradicting this study's results. According to Vanderhaeghen et al. (2014) and Nocera et al. (2021), the ability to hemolyze and ferment mannitol possessed by CoNS bacteria will differ for each species.

Culture results on BAP and MSA media from 54 isolates showed results that led to several species of CoNS bacteria. 3 isolates (5.56%) were γ-hemolytic and could not ferment mannitol, leading to *Staphylococcus epidermidis*, while 18 isolates (33.33%) were γ-hemolytic and fermented mannitol, leading to *Staphylococcus sciuri, Staphylococcus equorum,* and *Staphylococcus hominis*. 5 isolates (9.26%) were β-hemolytic and could not ferment mannitol, leading to *Staphylococcus schleiferi*, while 28 isolates (51.85%) were β-hemolytic and fermented mannitol, leading to *Staphylococcus aureus, Staphylococcus haemolyticus, Staphylococcus saprophyticus* and *Staphylococcus xylosus* (Tabel 2).

The formation of a hemolysis zone by bacteria occurs because bacteria produce exoproteins, namely hemolysin, a toxin (Nasaj et al. 2020). Bacteria produce hemolysin as a form of self-defense from the host's immune system and release iron from red blood cells, which is needed as a source of energy for growth (Divyakolu et al. 2019). Research Heo et al. (2020), stated that the production of hemolysin in CoNS bacteria will differ in each strain of its species due to various

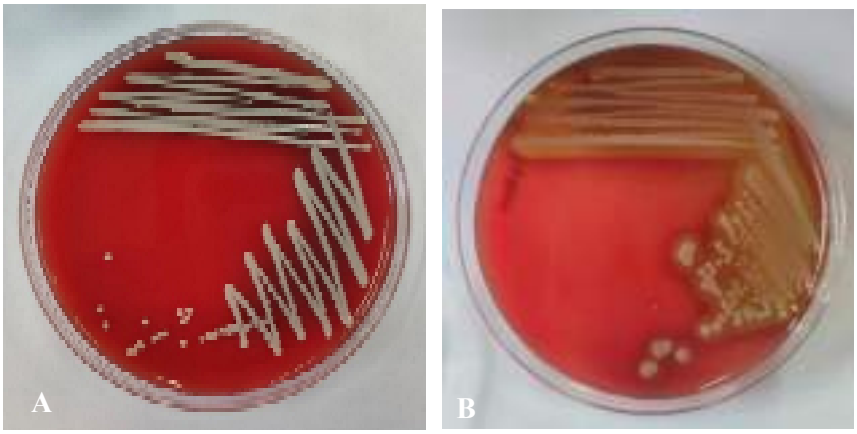

Figure 2. Coagulase-negative *Staphylococci* (CoNS) Colonies on Blood Agar Plate (BAP) Media. (A) γ-hemolysis colonies, (B) β-hemolysis colonies. (Personal Documentation).

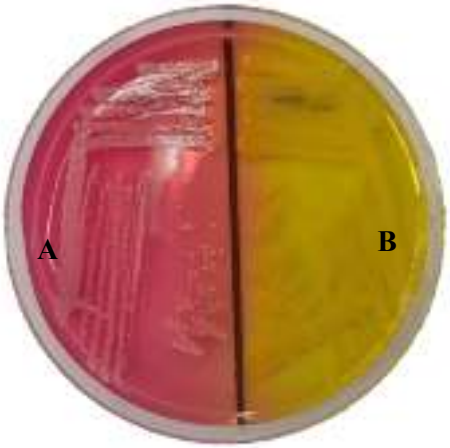

Figure 3. Coagulase-negative *Staphylococci* (CoNS) Colonies on Mannitol Salt Agar Plate (MSA) Media. (A) negative mannitol fermentation, (B) positive mannitol fermentation. (Personal Documentation)

Table 2. Species of coagulase-negative *Staphylococci* (CoNS) bacteria based on the results of hemolysis and mannitol fermentation zones from stored isolates from milk of dairy cattle suffering from subclinical mastitis

Results	Number	Suspected CoNS Bacteria
γ-hemolysis and MSA (-)	3	*Staphylococcus epidermidis* (Pinheiro et al. 2015)
γ-hemolysis and MSA (+)	18	*Staphylococcus sciuri* (Beims et al. 2016; Cirkovic et al. 2017) *Staphylococcus equorum* (Thakur et al. 2017) *Staphylococcus hominis* (Thakur et al. 2017)
β-hemolysis and MSA (-)	5	*Staphylococcus schleiferi* (Yarbrough et al., 2018)
β-hemolysis and MSA (+)	28	*Staphylococcus aureus* (Thakur et al. 2017) *Staphylococcus haemolyticus* (Pinheiro et al. 2015) *Staphylococcus saprophyticus* (Ayeni et al. 2017) *Staphylococcus xylosus* (Jeong et al. 2016)

β= Betta, γ= Gamma, MSA= Mannitol Salt Agar

hemolysin genes, namely hld, hlg, hlb, and hla. According to Azih & Enabulele (2013), the *Staphylococcus haemolyticus* bacterial strain is a CoNS bacterium that is predominant in producing hemolysin, while the CoNS bacterial strain that tends not to have the ability to hemolyze is *Staphylococcus epidermidis*.

The ability to produce hemolysin is one of the virulence factors of bacteria (Motamedi et al. 2018). Hemolysin plays an important role in infectious processes caused by bacteria because this toxin has a cytotoxic effect and can cause lysis in eukaryotic cells (Pakshir et al. 2017). Hemolysin can cause tissue damage and activation of the inflammatory response in the host (Zhao et al. 2020). Mannitol salt agar (MSA) is a differential media for bacteria (Boipai et al. 2020). This media contains about 7.5 – 10% salt (NaCl), so only bacteria with a high salt tolerance can grow, such as *Staphylococcus* sp. (Urip et al. 2022). Positive results on MSA media were obtained because bacteria fermented mannitol into organic acids, thus changing the pH indicator, namely phenol red in the media, to bright yellow (Rahmi et al. 2015). Bacteria's ability to ferment mannitol is regulated by the enzyme mannitol-1-phosphate dehydrogenase (M1PDH) (Virgianti et al. 2019).

CoNS bacteria are normal microflora of mammals' skin and mucous membranes (Thilakavathy et al. 2015). In dairy cows, this bacteria is commonly found at the tip of the teats, with the predominant species including *Staphylococcus haemolyticus* and *Staphylococcus equorum* (De Visscher et al. 2016). *Staphylococcus epidermidis* and *Staphylococcus aureus* are normal bacterial species on human skin, but in dairy cows, these bacteria are among the most common pathogenic bacteria isolated on the skin of the teat area (Chotigarpa et al. 2018; Brown and Horswill 2020). *Staphylococcus hominis* is another CoNS bacterium that belongs to the normal microflora of human skin (Azimi et al. 2020).

Infection by CoNS bacteria in cases of subclinical mastitis in dairy cows can occur during the milking process. Bacterial colonies on the skin can invade the mammary glands during this process (Chotigarpa et al. 2018). Bacteria can come from the milking hands or the equipment used because bacteria can form biofilms and tick and survive on the hands and equipment (El-Jakee et al. 2013). Decreased host resistance can also trigger an imbalance in the normal microflora in the body, which can lead to infection (Purwanti et al. 2018).

Detection of biofilm formation

Total of 49 out of 54 isolates (90.74%) showed positive results to form biofilms, while 3 isolates (5.56%) showed negative results when tested with the CRA and TT methods. 5 out of 54 isolates (9.26%) showed negative results when tested with the CRA method, but 49 isolates (90.74%) showed positive results with the same test method. More positive results were obtained with the TT test method, namely 51 isolates (94.44%), with fewer negative results, namely 3 isolates (5.56%) (Tabel 3). Reliability or The Cohen's Kappa coefficient (κ) value between the CRA and TT methods obtained in this study was 0.732, so it can be said that both methods are reliable.

The results in CRA test method in this study has almost the same results as the study of Gurler et al. (2022) and Abed et al. (2022). Differences in the percentage of positive results can be caused by differences in temperature or incubation time and the species of CoNS bacteria detected.Optimal positive results were obtained after incubation for 72 hours at 37 °C, according to the study of Cho et al. (2022). During incubation, the pH of the media decreased due to polysaccharides produced by bacterial metabolism and the degradation of sucrose. The polysaccharide then reacts with the Congo red indicator in the media, causing the pigmented bacterial colonies to turn black (Figure 4A) (HRV et al. 2016). The sucrose content in the media affects the production of EPS (extracellular polymeric substances) in bacteria. When the sucrose concentration is low, the diffusion of black pigment from the colonies will also be reduced (Normanita 2020).

The detection of biofilm formation using the TT method showed positive results in 51 of 54 samples (94.44%). This study has almost the same results as Raksha et al. (2019) and Schönborn et al. (2017). The difference in the percentage of positive results could be due to differences in temperature, incubation time, and the species of CoNS bacteria detected. Optimal positive results were obtained after incubation for 30 hours, according to research by Kusumaningrum et al. (2020). The biofilm formed by bacteria will adhere (adhesion) to

Table 3. Concordance between CRA and TT methods in detecting biofilm formation capability of coagulase negative *Staphylococci* (CoNS) isolate milk of dairy cattle suffering from subclinical mastitis

Method		*Congo Red Agar*		Total
		Positive	Negative	
Test Tube	Positive	49	2	51
	Negative	0	3	3
Total		49	5	54

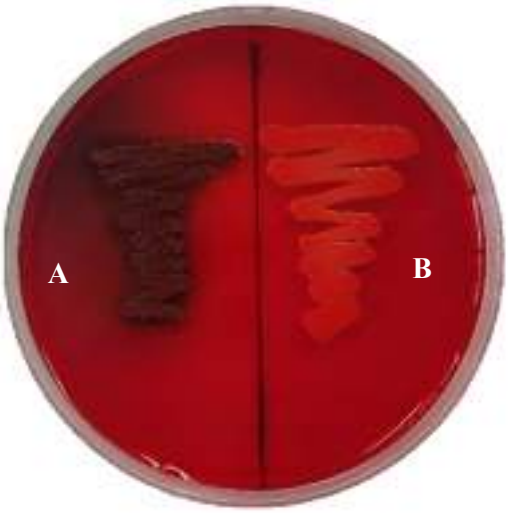

Figure 4. Coagulase-negative *Staphylococci* (CoNS) Colonies on Congo Red Agar (CRA). (A) positive biofilm, (B) negative biofilm. (Personal Documentation)

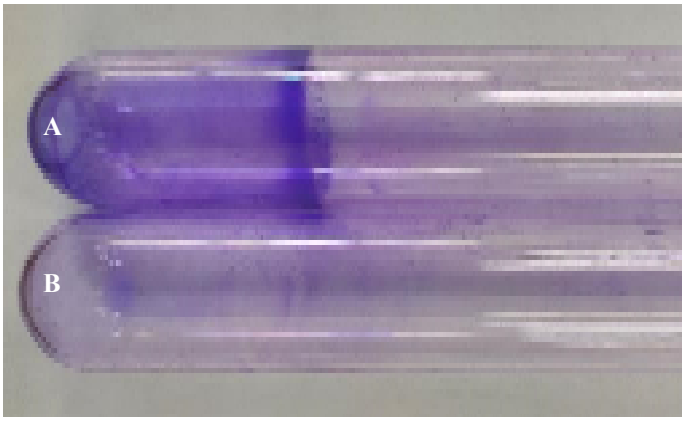

Figure 5. Biofilm Detection Test Results on Coagulase-negative *Staphylococci* (CoNS) Bacteria with the *Test Tube* (TT) Method. (A) positive biofilm, (B) negative biofilm. (Personal Documentation)

the tube wall so that when stained with crystal violet (Figure 5A), a ring will form at the bottom of the tube, and a bluish-purple inner layer of the tube (Tariq et al. 2021). This method uses Crystal violet as a dye because it can bind to negatively charged molecules to color the bacteria and the resulting matrix (Amador et al. 2021).

CoNS bacteria are commensal bacteria on human and animal skin. They are considered less pathogenic because they do not have virulence factors like CoPS bacteria and are rare in clinical pathology discussions (Argemi et al. 2019). The ability to form biofilms is an important virulence factor for CoNS bacteria, and this ability makes bacteria attach to abiotic and biotic layers and form defenses from host phagocytosis, chemotaxis, and antimicrobial agents (Shrestha et al. 2017; Manandhar et al. 2021). PIA regulates bacterial biofilm formation polysaccharide intercellular adhesin) encoded by the icaABCD (intercellular adhesion ABCD) gene (Kord et al. 2018). Biofilms that have formed are difficult to destroy, so treatment requires high doses of antibiotics or removing the infected part (Di Somma et al. 2020).

CONCLUSION

Detection of the profile of biofilm formation in CoNS bacteria derived from milk from subclinical mastitis dairy cows with a positive CMT value of 2 (++) using CRA, and TT on isolates showed that 51 out of 54 isolates (94.44%) were positive for forming biofilms.

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