

Prevalence and antimicrobial resistance in *Streptococcus agalactiae* isolated from raw milk in Pasuruan and Lumajang districts, East Java, Indonesia

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Manuscript received: 20 August 2022. Revision accepted: 27 September 2022.

Abstract. Permatasari DA, Anggraeni F, Budiarto, Meles DK, Hamid IS, Puspitasari Y, Effendi MH, Khairullah AR, Wardhana DK, Ugbo EN. 2022. Prevalence and antimicrobial resistance in *Streptococcus agalactiae* isolated from raw milk in Pasuruan and Lumajang districts, East Java, Indonesia. *Biodiversitas* 23: 5050-5055. Milk and dairy products are nutritionally important in the diet worldwide. The microbiological quality of raw milk is essential for the quality of the final dairy product. The presence of *Streptococcus agalactiae* is frequently associated with high somatic cell counts in milk and decreased milk yield. *Streptococcus agalactiae* infections have major consequences for public health. This study aimed to identify the prevalence and antimicrobial resistance of *Streptococcus agalactiae* isolated from raw milk in Pasuruan and Lumajang, East Java, Indonesia. Raw milk samples were collected from three sub-districts in Pasuruan (Tutur, Grati and Purwosari) and three sub-districts in Lumajang (Senduro, Karangbendo, and Tekung). California mastitis test (CMT) was performed to confirm the presence of somatic cells, *S. agalactiae* was identified using standard microbiological methods, and antibiotic resistance was determined by Kirby-Bauer disc diffusion method using Mueller-Hinton agar. Results showed that 89 (79.46%) out of 112 dairy cows were positive for CMT, which indicated the presence of mastitis. Bacteria isolation was performed on CMT-positive samples and four samples were showed the presence of *S. agalactiae* (4.49%). Based on the sensitivity test against various antibiotics, it was observed that *S. agalactiae* isolates were resistant to ampicillin (75%), and erythromycin (50%), and were sensitive to cefotaxime (100%), tetracycline (75%), and chloramphenicol (100%). This research reported that *Streptococcus agalactiae* organism is one bacterium that is implicated in mastitis infections. Therefore, monitoring the antibiotic susceptibility profiles of this organism is needed in veterinary medicine, in order to make an appropriate choice of drug for treatment, improve high cure rates and minimize the increase of drug resistance.

Keywords: Antimicrobial resistance, California Mastitis Test, public health, raw milk, *Streptococcus agalactiae*

INTRODUCTION

Important nutrients in diets around the world are largely supported by milk and dairy products. The microbiological quality of raw milk is very important for the quality of the final milk product (Åkerstedt 2012). Cases of mastitis in dairy cows often use antimicrobial drugs, where mastitis is one of the most common infectious diseases in dairy cows. The bacteria involved in bovine mastitis are classified as infectious or environmental pathogens based on their epidemiological relationship to the disease. Streptococcal species are the main mastitis pathogens, along with *Staphylococcus aureus* and coliforms. *Streptococcus agalactiae* is associated with cattle and is well adapted to the mammary glands (Minst et al. 2012). Therefore, it is the only mastitis pathogen that can be removed from the

herd using blanket therapy with penicillin or its derivatives (Boonyayatra 2020). Subclinical mastitis is difficult to detect visually. The signs are inflammation of the udder and abnormal milk. Finding new mastitis markers that can identify the condition at an early stage is critical for milk production (Pongthaisong et al. 2016). Generally, increasing the number of milk somatic cells and bacterial culture are the methods used to detect subclinical mastitis (Radostits et al. 2007).

An increase in the number of somatic cells in milk in cases of mastitis is a common and expensive disease in dairy cows that shows varying degrees of severity and reduces the quantity and quality of milk produced (Heikkilä et al. 2012; Rollin et al. 2015). *Streptococcus agalactiae*, a member of the Lancefield B group, is an important cause of chronic infectious bovine mastitis (Herlina et al. 2015). It

also causes mastitis and invasive disease in camels and occasionally causes disease in dogs, cats, fish and hamsters. This pathogen is often associated with high somatic cell counts in milk and decreased milk production. *Streptococcus agalactiae* infection has major public health effects, as it can cause neurological problems in new born humans and endometritis and infertility in mothers. Changes in the diversity and abundance of these bacteria affect udder health and physiology, milk production and quality, and milk sustainability (Mansor et al. 2013; Yang et al. 2016). Studies have attempted to identify unknown metabolites or degradation products in milk from cows with subclinical mastitis. The specific effects of *S. agalactiae* on the milk microbiota have also not been investigated in controlled longitudinal studies (Tong et al. 2019).

Milk proteins are categorized by their respective defence mechanism: inflammation-related or an antimicrobial response to mastitic pathogens (Smolenski et al. 2007; Effendi et al. 2019a). Mastitis therapy is commonly initiated before susceptibility testing of the pathogen (Hendriksen et al. 2008). Treatment of streptococcal mastitis often uses β -lactam and macrolide antimicrobial classes (Kalmus et al. 2011; Harijani et al. 2020). Because the emergence of resistant pathogens is of growing concern in veterinary medicine, performing susceptibility tests during the bacteriological examination of mastitis milk samples is an important basis for the selection of the appropriate chemotherapeutic agents (Schwarz et al. 2003; Ramandinianto et al. 2020).

Antimicrobial resistance is currently an area of interest in human and veterinary medicine, underscoring the importance of antimicrobial resistance monitoring to obtain information on resistance levels and observe the effects of interventions (Minst et al. 2012; Tyasningsih et al. 2019). The presence of multidrug-resistant (MDR) isolates refers to strains that are resistant to three or more types of antimicrobial drugs simultaneously (Ansharieta et al. 2021). Emerging antimicrobial resistance in clinical, community and veterinary environments has become a threat to public health worldwide (Li et al. 2020; Khairullah et al. 2022). Previous research on the antimicrobial resistance of *S. agalactiae* has been conducted in Surabaya. The results are sensitive to erythromycin and resistant to ampicillin, penicillin, and tetracycline (Karina 2016). Another report from Campo das Vertentes, Brazil showed that *S. agalactiae* was resistant to ampicillin (37%), chloramphenicol (44%), and tetracycline (77%) (Mesquita et al. 2019). This study aimed to identify the prevalence and antimicrobial resistance of *S. agalactiae* from raw milk in Pasuruan and Lumajang, East Java, Indonesia.

MATERIALS AND METHODS

Sample collection

Milk samples were collected from three sub-districts in Pasuruan (Tutur, Grati, and Purwosari) and also three sub-districts in Lumajang (Senduro, Karangbendo, and Tekung) of East Java Province, Indonesia. A total of 112 lactating

cows were used for milk sampling. The samples were taken between March to May 2022. Before sampling, the nipples were cleansed with cotton soaked in 70% ethanol and the first milk was discarded. Approximately, 10 mL of raw milk was collected from each teat and samples from one cow were pooled together as one sample (Effendi and Harijani 2017). Milk samples were kept cool during transportation using ice packs and were stored in the refrigerator at (4°C) for a maximum of 12 hrs before culturing.

California Mastitis Test (CMT)

California Mastitis test (CMT) is a simple indicator of the somatic cell count in milk (Björk 2013). The test was carried out by taking 2 mL of milk placed on a paddle and then reacting with CMT reagent as much as 2 mL of milk from each udder quarter (Khairullah et al. 2019). CMT scores of all samples were determined by using solution and equipment. CMT is graded visually by observing the presence or absence of changes in milk viscosity. The result is categorized as (-) negative by no precipitate in milk, (+1) by a little sediment in milk, (+2) by clear deposits but the gel has not yet formed, (+3) by the thickening of the mixture and begins to form the gel, and (+4) by the formation of gel that causes the surface to become convex (Khairullah et al. 2019).

Isolation and identification of *Streptococcus agalactiae*

Milk samples were spread onto nutrient agar (NA) plates and plates were incubated for 24 hrs at 37°C. The isolated pure culture was characterized using Gram staining. The biochemical test was carried out using a catalase test. The catalase test was carried out by dripping 3% hydrogen peroxide (H₂O₂) on bacterial colonies placed on the object glass's surface (Mustafa 2014). The isolates were sub-cultured onto blood agar (BA) plate (Himedia) to identify the *Streptococcus* with characteristics of α -hemolysis, β -hemolysis, or without hemolysis/ γ -hemolysis. Isolates were identified as *Streptococcus* if they were Gram-positive cocci with a negative catalase reaction. *Streptococcus* colonies with β -hemolysis were characterized using Christie, Atkins, Munch-Peterson test (CAMP) to identify *S. agalactiae* strains (Ahmadi et al. 2009).

Antibiotic sensitivity confirmation test

The antimicrobial susceptibility test was carried out using the Kirby-Bauer disc diffusion method using Mueller-Hinton agar (Effendi and Harijani 2017). All discs used in the disc diffusion assay were obtained from Oxoid, UK, in the following concentrations: cefotaxime (CTX 30 g), ampicillin (AMP 10 g), tetracycline (TE 30 g), erythromycin (E 15 g) and chloramphenicol (C 30 g). Culture turbidity was adjusted to the 0.5 McFarland standard. A sterile cotton swab was dipped in the suspension and applied evenly to the entire surface of the Mueller-Hinton agar. Antibiotic discs were placed on the inoculated plate surface and incubated at 37°C for 16-18 hrs. The diameter of the inhibition zone was measured in millimeters and interpreted as susceptible, moderate, and resistant (Permatasari et al. 2020). Evaluation of specimens for antibiotic resistance was performed according to the

Clinical and Laboratory Standards Institute (CLSI 2020) guidelines.

RESULTS AND DISCUSSION

California Mastitis Test (CMT)

The results of this study showed that 89 (79.46%) out of 112 dairy cows in Pasuruan (Tutur sub-district, Grati sub-district, and Purwosari sub-district) and Lumajang (Senduro sub-district, Karangbendo sub-district, and Tekung sub-district) were positive for CMT, which indicates the presence of mastitis (Table 1). This result was similar to a previous study showing a high evidence rate of subclinical mastitis in East Java. Previous reports showed that the prevalence rate of subclinical mastitis in dairy farms around East Java reached 85.33% (Effendi et al. 2019b).

Isolation and identification of *Streptococcus agalactiae*

Bacterial identification was performed on CMT-positive samples using morphology, Gram staining, catalase, and CAMP tests. Four samples were positive for *S. agalactiae* (4.49%). Based on the results of isolation on NA media there are diverse cultures, but only a few colonies have the characteristics of *S. agalactiae*, which is round, small colonies with varying diameters (0.5 - 2.0 mm), smooth, transparent, and convex (Ahmadi et al. 2009). Based on the results of a negative catalase test, *S. agalactiae* does not form a bubble when dripped hydrogen peroxide is on object glass because this bacterium does not produce catalase enzyme to hydrolyze hydrogen peroxide into water and gas bubbles. CAMP (Christie, Atkins, Munch-Peterson) test was performed to see the *S. agalactiae* increasing hemolytic activity in Staphylococcal β -toxin to form arrow-like signs on CAMP reactions (Khairullah et al. 2019).

Antibiotic sensitivity test

An antibiotic sensitivity test using Kirby-Bauer disc diffusion method (Figure 2) was performed and the results were analyzed (Table 3). The number of positive samples from the CAMP-test

were 4 (4.49%). The percentage of isolates classified as resistant, intermediate or sensitive was used to determine the overall resistance prevalence and disaggregated by sample code. A high percentage of *S. agalactiae* resistance was observed on ampicillin (75%), erythromycin (50%) antibiotics and sensitivity to cefotaxime (100%), tetracycline (75%), and chloramphenicol (100%).

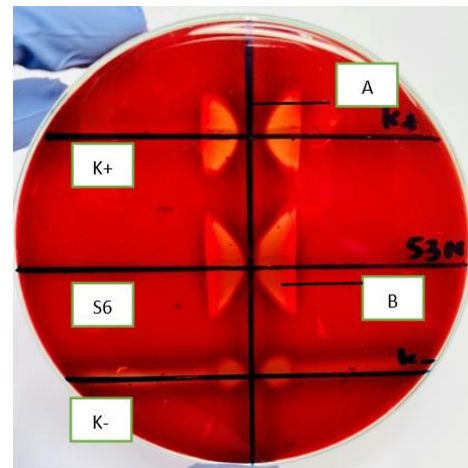


Figure 1. Christie-Atkins-Munch-Peterson (CAMP) test result from raw milk sample with subclinical mastitis. Information: A: *Staphylococcus aureus* bacteria; B: arrow marks that show CAMP test results; K+: Positive control, *Streptococcus agalactiae*; S11: sample; K-: negative control, *Streptococcus pyogenes*

Table 1. Data of California Mastitis Test

Location		No. of samples	No. of positive CMT	Percentage
Pasuruan	Tutur	22	11	50.00
	Grati	25	23	92.00
	Purwosari	5	5	100.00
Lumajang	Senduro	39	31	79.48
	Karangbendo	16	14	87.50
	Tekung	5	5	100.00
Total		112	89	79.46

Table 2. Sensitivity test of *Streptococcus agalactiae* according to inhibition zone diameter

Sample code	Antibiotic resistance zone diameter (mm)				
	Cefotaxime	Ampicillin	Tetracycline	Chloramphenicol	Erythromycin
Kb-8	42.12 (S)	32.28 (S)	36.94 (S)	28.18 (S)	29.24 (S)
S-6	30.62 (S)	15.86 (R)	22.72 (I)	30.42 (S)	6.32 (R)
S-27	29.52 (S)	21.84 (R)	23.64 (S)	29.64 (S)	30.64 (S)
T-6	43.14 (S)	23.82 (R)	28.26 (S)	35.14 (S)	6.32 (R)

Note: R= Resistant I= Intermediate S= Sensitive

Table 3. Results of *Streptococcus agalactiae* sensitivity test according to the number of samples

Milk sample	Criteria	Antibiotic type				
		Cefotaxime	Ampicillin	Tetracycline	Chloramphenicol	Erythromycin
4	S	4 (100%)	1 (25%)	3 (75%)	4 (100%)	2 (50%)
	I	0 (0%)	0 (0%)	1 (25%)	0 (0%)	0 (0%)
	R	0 (0%)	3 (75%)	0 (0%)	0 (0%)	2 (50%)

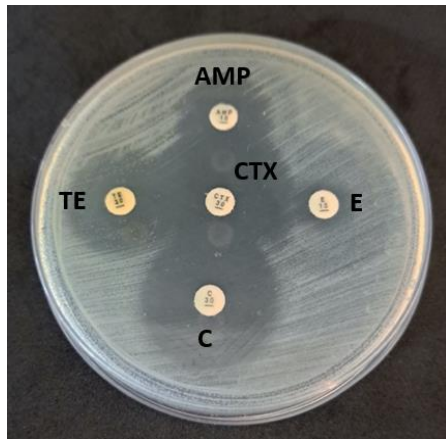


Figure 2. Antibiotic sensitivity test using Kirby-Bauer disc diffusion method on Mueller-Hinton agar. Information: ampicillin (AMP), tetracycline (TE), cefotaxime (CTX), erythromycin (E), chloramphenicol (C)

Discussion

Mastitis caused by streptococci is a superficial infection because the pathogen remains in the milk ducts. *Streptococcus agalactiae* is an udder pathogen and is highly contagious. It spreads mainly during milking (Björk 2013). Subclinical mastitis in dairy cattle is a major problem and often causes higher economic losses for farmers. This is one of the main reasons for low yields and poor milk quality and ranks first among diseases that cause heavy losses to owners (Kumari et al. 2018). Mastitis is defined as intramammary infection with *S. agalactiae*, known as Group B Streptococcus (GBS), and often results in chronic subclinical mastitis (Jørgensen et al. 2016). Prevention measures, improved management, and better sanitation have reduced the number of cases of infectious mastitis and have led to changes in the etiology of the disease in the last decade (Middleton et al. 2014). Most cases of mastitis are caused by several common bacterial pathogens, namely *Staphylococcus* sp., *Streptococcus* sp., coliforms, and *Actinomyces pyogenes* (Sharma et al. 2010).

In this study, the California mastitis test (CMT) was used to determine quarterly subclinical mastitis prevalence and herd rates for individual cattle and their herds. A high prevalence of mastitis in raw dairy cow milk was recorded in this study. CMT has been the only reliable cow-side screening test for subclinical mastitis since last 50 years. Although it does not identify the type of bacteria that cause mastitis, the CMT is useful in identifying quarters that have high somatic cells (Argaw 2016). *Staphylococcus aureus* and *S. agalactiae* are referred to as contagious udder pathogens as they are bound to the bovine udder or the cow and are mainly transmitted from cow to cow (Björk 2013). This study observed that 89 (79.46%) out of 112 dairy cows in Pasuruan (Tutur sub-district, Grati sub-district, and Purwosari sub-district) and Lumajang (Senduro sub-district, Karangbendo sub-district, and Tekung sub-district) were positive for CMT. The advantages of using the CMT method are that an inflammatory reaction of the udder can be known as early as possible, less treatment cost is easy to perform, and can be done for multiple milk samples

simultaneously (Effendi et al. 2019b). A high proportion of subclinical mastitis was found on a majority of the visited farms. The high prevalence might be an effect of the observed poor milking hygiene (Sraïri et al. 2009) and the traumatic strip milking technique being practiced on all farms. However, it has not yet been shown that the strip milking technique is causing higher somatic cells than other hand milking techniques (Millogo et al. 2012).

The results of bacteriological cultures in this study do not reflect the general definition of subclinical mastitis. In other studies of subclinical mastitis, Coagulase-Negative Staphs (CNS) was also the most common finding (Abrahmsén et al. 2012; Byarugaba et al. 2008). Argaw (2016) states that knowledge of the infection status of the mammary glands, however, can also be of great help to prevent pathogen transmission by diagnosing the reservoir at an early stage. Another public health problem associated with mastitis is antibiotic residue in milk due to the extensive use of antibiotics in the treatment and control of the disease. Antibiotic residues in food can cause severe reactions in people who are allergic to antibiotics and, at low levels, can cause sensitization in normal individuals and the development of antibiotic-resistant bacterial strains.

Streptococcus agalactiae resistance test showed fairly high resistance to ampicillin. This result is in contrast to antimicrobial susceptibility test against *S. agalactiae* in the Campo das Vertentes region, Brazil which showed that this bacterium is sensitive to ampicillin (63%) (Mesquita et al. 2019). Ampicillin resistance is possible to develop with cross-resistance. Cross-resistance is a specific drug resistance that can occur in other drugs with a retention mechanism (Effendi et al. 2018). Ampicillin is broad-spectrum penicillin that maintains the antibacterial spectrum of penicillin and is susceptible to hydrolysis by β -lactamases. The increased resistance to macrolides among Group B *Streptococcus* isolates is a therapeutic problem for those allergic to β -lactams (Lee et al. 2015).

In both human and veterinary medicine, antimicrobial resistance in bacteria is a problem on the rise. It is stated by the World Organization for Animal Health (WOAH) that the human, animal, and plant sectors must take responsibility for reducing the development of resistant pathogens. Prudent use of antimicrobial therapies and monitoring of antimicrobial susceptibility of bacterial flora in animals are two examples of recommendations to achieve this goal (OIE 2016). Antimicrobial therapy should be guided by the antimicrobial susceptibility test and the necessity for the continuous monitoring of antimicrobial susceptibility profiles should also be emphasized, as well in general (Guo et al. 2019). The resistance is due to one of four general mechanisms: (1) inactivation of antibiotic by β -lactamase, (2) modification of target PBPs, (3) impaired penetration of drug to target PBPs, and (4) efflux (Katzung et al. 2012).

This study indicated that *S. agalactiae* showed 50% resistance to erythromycin. Erythromycin resistance in Streptococci is caused by the wide distribution of the erythromycin ribosome methylase (*erm*) gene and the possible development of complete cross-resistance. The resistance can arise because there is a change in the

erythromycin L4 or L12 protein-coding gene in the 50S bacterial ribosome subunit, resulting in a decrease in erythromycin affinity for ribosomes. Erythromycin-resistant Gram-positive cocci should not be treated with 16-limbed macrolides (macrolides with a 16-membered lactone circle) (Minst et al. 2012). *Streptococcus agalactiae* infections in both humans and bovines are treated by the administration of antibiotics. The increase in antibiotic resistance among bacterial populations is often due to the extensive use of antibiotics in medicine and animal husbandry (Effendi et al. 2018). Monitoring the antibiotic susceptibility profiles is needed for a more careful selection of antibiotics in order to improve high cure rates and minimize the increase of drug resistance.

In conclusion, this research reported the 4.49% prevalence of *S. agalactiae* organisms in raw milk from dairy cows. It was also observed that *S. agalactiae* is one of the bacteria that is implicated in mastitis infections and reported the presence of mastitis in 89 (79.46%) out of 112 raw dairy cows' milk studied. High levels of resistance to *S. agalactiae* were observed in ampicillin, followed by erythromycin antibiotics. The presence of antibiotic resistance as observed in *S. agalactiae* is a threat to public and animal health. The impact of these conditions limited and complicated the drug of choice in *S. agalactiae* infection such as mastitis cases on the dairy farm. Therefore, policy and veterinarian supervision is important to implement disease control, treatment, and management of antibiotic usage in animal husbandry.

ACKNOWLEDGEMENTS

This study was supported by the Rector of Universitas Airlangga, Surabaya, Indonesia for providing funding for this study by *Penelitian Unggulan Fakultas*, Universitas Airlangga with grant number: 1405/UN3.1.6/PT/2021, and also the authors would like to thank Pasuruan and Lumajang Dairy Farm for their cooperation.

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