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Multidrug-Resistant *Staphylococcus aureus* Isolated from Cattle Milk in Indonesia

(STAPHYLOCOCCUS AUREUS RESISTAN TERHADAP BERBAGAI ANTIMIKROBA YANG DIISOLASI DARI SUSU SAPI DI INDONESIA)

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Abstract

Staphylococcus aureus (*S. aureus*) is one of the common causes of bovine mastitis worldwide. Inappropriate use of antimicrobials in cattle farms contributes to the increase of the antimicrobial resistance of *S. aureus*. This study aimed to investigate the antimicrobial resistance profiles of *S. aureus* isolated from cattle milk. Twelve *S. aureus* isolated from cattle milk in Bogor, Indonesia, were tested against seven antimicrobials using Kirby-Bauer disk diffusion susceptibility test. The results revealed that 58.3 % of isolates were multidrug-resistant *S. aureus*. The *S. aureus* isolates were resistant to penicillin G (66.7 %), ampicillin (66.7 %), vancomycin (58.3 %), and bacitracin (58.3 %). Most of the isolates were susceptible to ciprofloxacin (91.7 %), gentamicin (66.7 %), and chloramphenicol (66.67 %). If clinically indicated, treatment of mastitis should consider the antimicrobial susceptibility of the causative agent.

Keywords: *Staphylococcus aureus;* antimicrobials; resistance; bovine mastitis

Abstrak

Staphylococcus aureus (*S. aureus*) merupakan salah satu penyebab utama mastitis pada sapi di seluruh dunia. Penggunaan antimikroba secara tidak tepat di peternakan sapi menyebabkan peningkatan bakteri yang resistan terhadap antimikroba. Penelitian ini bertujuan untuk menginvestigasi profil resistansi antimikroba *S. aureus* yang diisolasi dari susu sapi. Dua belas isolat *S. aureus* yang diisiolasi dari sampel susu sapi di Bogor, Indonesia, diuji terhadap tujuh antimikroba menggunakan metode agar difusi menurut Kirby-Bauer. Hasil penelitian menunjukkan bahwa 58.3 % isolat merupakan *S. aureus* resistan terhadap beberapa antimikroba. Isolat *S. aureus* yang diuji resistan terhadap penisilin G (66,7 %), ampisilin (66,7 %), vankomisin (58,3 %), dan basitrasin (58,3 %). Sebagian besar isolat sensitif terhadap siprofloksasin (91,7 %), gentamisin (66,7 %), dan kloramfenikol (66,67 %). Pengobatan mastitis harus mempertimbangkan resistansi antimikroba dari bakteri penyebab penyakit.

Kata kunci: *Staphylococcus aureus;* antimikroba; resistan; mastitis

INTRODUCTION

Bovine mastitis is one of the most common diseases affecting the quantity and quality of world milk production (Ruegg, 2017; Wang *et al.,* 2022). Mastitis incurs various costs, including decreased milk production, condemnation of milk due to antibiotic residues, veterinary expenses, and culling of chronically infected cattle (Nurhayati and Martindah, 2015; Abebe *et al.,* 2016). *Staphylococcus aureus* (*S. aureus*), a Gram-positive bacterium, is one of the common causes of bovine mastitis worldwide (Ruegg, 2017, Abebe *et al.,* 2016; Chaalal *et al.*, 2016).

A previous study reported that the treatment of subclinical and clinical mastitis in Indonesia is still not effective because it generally uses broad-spectrum antimicrobials without conducting a specific analysis of the causative agent

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(Nurhayati and Martindah, 2015). Antimicrobials often used in farms include penicillin, tetracycline, aminoglycosides, and macrolides (Meutia *et al.,* 2016). Inappropriate use of antimicrobials causes antimicrobial resistance, which is now a global problem. Previous studies reported that antimicrobial-resistant bacteria were isolated from cattle in Indonesia (Klobongona *et al.,* 2019; Tyasningsih *et al.,* 2021; Rotinsulu *et al.,* 2022). In order to maximise the benefits of antimicrobial stewardship, continuous monitoring of bacterial pathogens for antimicrobial resistance is essential. The use of antimicrobials to treat bacterial infections caused by *S. aureus* in cattle farms encourages the investigation of antimicrobial resistance of *S. aureus* isolated from cattle milk.

This study aimed to investigate the antimicrobial resistance patterns of *S. aureus* isolated from cattle milk against penicillin G, ampicillin, vancomycin, gentamicin, bacitracin, ciprofloxacin, and chloramphenicol. Furthermore, the results of this study can help clinicians in selecting appropriate antimicrobials for treating mastitis caused by *S. aureus*.

RESEARCH METHODS

Materials and Equipment

Materials used in this study were twelve *S. aureus* isolates, crystal violet, lugol's iodine, safranin, H_2O_2 3%, glucose fermentation test, 0.5 McFarland standard, blood agar, tryptic soy agar (TSA), mannitol salt agar (MSA), Mueller-Hinton agar (MHA), antimicrobial discs penicillin G (10 IU), ampicillin (10 µg), vancomycin (30 µg), gentamicin (10 µg), bacitracin (10 IU), ciprofloxacin (5 µg), and chloramphenicol (30 µg). Equipment used in this study were anaerobic jar, sterile cotton swab, inoculation needle (öse), forceps, bunsen burner, vortex mixer, 37 ^oC aerobe incubator, sterile cabinet, refrigerator, and ruler.

Identification of *Staphylococcus aureus*

Isolates used in this study were twelve *S. aureus* isolates from the bacterial culture collection of the Laboratory of Bacteriology, Division of Medical Microbiology, School of Veterinary Medicine and Biomedical Sciences, IPB University, Bogor, Indonesia. All *S. aureus* were isolated from raw milk of cattle that had mastitis in Bogor, Indonesia. All isolates were re-assessed for species identification using standard bacterial culture procedure (Carter and Cole 1990; FDA 2005). Briefly, tested bacterial cultures were inoculated on blood agar plates and then incubated aerobically for 24 hours at 37 ^oC. Agar plates were examined for colony morphologic features, including colony shape, size, colour, and haemolytic characteristics. Single colonies with macroscopic phenotype suspected as Staphlycoccocci were subcultured on TSA and incubated for 24 hours at 37 ^oC. Further identification was performed using Gram staining and biochemical reactions. Only Gram-positive cocci bacteria arranged in clusters were included for the further biochemical test, consisting of catalase test, glucose fermentation test in microaerophilic condition, colony morphology on manitol salt agar, and coagulase test (Carter and Cole 1990; FDA 2005).

Antimicrobial Susceptibility Test

The antimicrobial resistance test of *S. aureus* was conducted using the Kirby-Bauer disk diffusion susceptibility test protocol according to Clinic Laboratory Standard Institute (CLSI 2020). Bacterial suspension equal to 0.5 McFarland standard was inoculated on MHA using a sterile *cotton swab* over the entire agar surface. Afterwards, seven antimicrobial discs with known concentrations (penicillin G, ampicillin, vancomycin, gentamicin, bacitracin, ciprofloxacin, and chloramphenicol) were placed on the MHA plate using sterile forceps. Agar plates were incubated aerobically at 37 ^oC for 24 hours. The antimicrobial susceptibility test was

performed in triplicates for each isolate. Interpretation of susceptible (S), intermediate (I), dan resistant (R) was conducted based on the size of the inhibition zone according to the breakpoints recommended by *Clinical and Laboratory Standards Institute* (CLSI 2020).

Data Analysis and Interpretation

Data were processed using Microsoft Excel 2016. Results were analyzed descriptively and visualised using tables and figures.

RESULTS AND DISCUSSION

Results

All twelve isolates were confirmed as *S. aureus* based on their macroscopic phenotype on the agar plate, microscopic morphology, and biochemical properties. All isolates formed double zone haemolysis on blood agar plates (Figure 1), were Gram-positive cocci in clusters, catalase positive, glucose positive, coagulase positive, and had yellow colonies on MSA (Figure 2).

The antimicrobial susceptibility test was conducted against seven antimicrobials belonging to various antimicrobials classes, namely penicillin G (beta-lactams), ampicillin (beta-lactams), vancomycin (glycopeptides), gentamicin (aminoglycosides**)**, bacitracin (polypeptides), ciprofloxacin (fluoroquinolones), and chloramphenicol (phenicols) (Figure 3).

The antimicrobial susceptibility test revealed that the tested *S. aureus* isolates had various antimicrobial resistance patterns. Seven isolates (58.3 %) were multidrug-resistant *S. aureus*. Interestingly, one isolate (SA08) was resistant to all tested antimicrobials, while another isolate (SA04) was susceptible to all tested antimicrobials (Table 1). Most isolates were resistant to penicillin G (66.7 %), ampicillin (66.7 %), vancomycin (58.3 %), and bacitracin (58.3 %). Almost all isolates were susceptible to ciprofloxacin (91.7 %), and more than half of the isolates were susceptible to gentamicin (66.7 %), and chloramphenicol (66.7 %) (Figure 4).

Discussion

Most of the isolates were resistant to the two tested beta-lactam antimicrobials, namely penicillin G and ampicillin. These results correlate with studies of bovine mastitis in Asia where 77.3 % *S. aureus* in China were resistant to penicillin G and ampicillin (Li *et al.*, 2009), and 66 % *S. aureus* in Korea were resistant to penicillin (Nam *et al.*, 2011). Additionally, 35.4 % and 15.1% of dairy cattle herds in Canada had antimicrobial-resistant *S. aureus* against penicillin G and ampicillin, respectively (Saini *et al.*, 2012). Both antimicrobials were previously commonly used to treat bacterial infections in cattle (Li *et al.*, 2009). However, the increase of *S. aureus* resistance against beta-lactams antimicrobials argues against their use in cattle with mastitis caused by *S. aureus*.

Methicillin is one important antimicrobial belonging to the beta-lactamase antimicrobial class (CLSI 2020). Methicillin-resistant *S. aureus* (MRSA) strain is resistant to most beta-lactam antimicrobials, which makes the treatment challenging (Fessler *et al*., 2010; Khairullah *et al*., 2020). MRSA associated with bovine mastitis was first described in 1975 (Devriese and Hommez, 1975). Currently, MRSA has been reported worldwide (Fessler *et al*., 2010; Nam *et al*., 2011; Khairullah *et al*., 2020). It would be interesting to investigate the antimicrobial susceptibility of the *S. aureus* isolates in this study against methicilin.

More than half (58.3 %) of the *S. aureus* isolates in this study were resistant against vancomycin. Vancomycin resistance was also reported by Sashidaran *et al.,* (2011) in Malaysia and Daka *et al.,*(2012) in Ethopia. In Ethiopia, 23.5 – 70 % of *S. aureus* isolates from cattle milk were resistant to vancomycin (Daka *et al.,* 2012). Furthermore, over half (58.3 %) of *S. aureus* isolates in this study were resistant to bacitracin. A study in Algeria reported that 70 % of methicillin susceptible *S.*

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aureus isolated from milk were resistant to bacitracin (Chaalal *et al.,* 2016).

In this study, most isolates were susceptible to ciprofloxacin, gentamicin, and chloramphenicol. These results correlate with a meta-analysis of bovine-associated *S. aureus* in China which reported that 63.8 %, 73.6 %, and 89.7 % *S. aureus* isolates were susceptible to quinolones, aminoglycosides, and phenicols, respectively (Wang *et al*., 2022). A study in Korea showed that 88.1 % *S. aureus* isolates were susceptible to gentamicin (Nam *et al.,* 2011), while another study in Germany reported that 68.7 % MRSA isolates were susceptible to gentamicin (Fessler *et al*., 2010). In Uganda, Staphylococci isolated from bovine mastitis cases were susceptible to ciprofloxacin and gentamicin (Kateete *et al.,* 2013). This result implies that these antimicrobials are still effective in treating bovine mastitis caused by *S. aureus*. However, antimicrobial stewardship should be considered in applying these drugs, especially for chloramphenicol which is a broad-spectrum antimicrobial.

CONCLUSION AND RECOMMENDATION

Conclusion

This study revealed that multidrug-resistant *S. aureus* isolates were isolated from cattle milk in Bogor Regency, Indonesia. These isolates were resistant to penicillin G (66.7 %), ampicillin (66.7 %), vancomycin (58.3 %), and bacitracin (58.3 %). However, most of the isolates were still susceptible to ciprofloxacin (91.7 %), gentamicin (66.7 %), and chloramphenicol (66.67 %). If clinically indicated, treatment of mastitis should consider the antimicrobial susceptibility of the causative agent.

Recommendation

Further study is necessary to determine antimicrobial-resistant *S. aureus* isolated from more samples and a wider area. Moreover, antimicrobial resistance genes should be determined, especially for multidrug-resistant strains.

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Table 1. Antimicrobial resistance patterns of twelve *Staphylococcus aureus* isolates from cattle milk

Isolate ID	Antimicrobial susceptibility*							Number of test results*		
	Pen G	Amp	Van	Gen	Bac	Cip	Chlor	S	I	R
SA01	R	R	R	I	R	S	S	2	1	4
SA02	R	R	S	S	S	S	S	5	0	2
SA03	S	S	S	R	S	S	S	6	0	1
SA04	S	S	S	S	S	S	S	7	0	0
SA05	R	R	R	S	R	S	S	3	0	4
SA06	I	S	S	S	S	S	S	6	1	0
SA07	R	R	R	S	R	S	S	3	0	4
SA08	R	R	R	R	R	R	R	0	0	7
SA09	R	R	R	S	R	S	I	2	1	4
SA10	R	R	R	S	R	S	I	2	1	4
SA11	S	S	S	S	S	S	I	6	1	0
SA12	R	R	R	R	R	S	S	2	0	5

Note: * Pen G: penicillin G, Amp: ampicillin, Van: vancomycin, Gen: gentamicin, Bac: bacitracin, Cip: ciprofloxacin, Chlor: chloramphenicol, S: susceptible, I: intermediate, R: resistant.

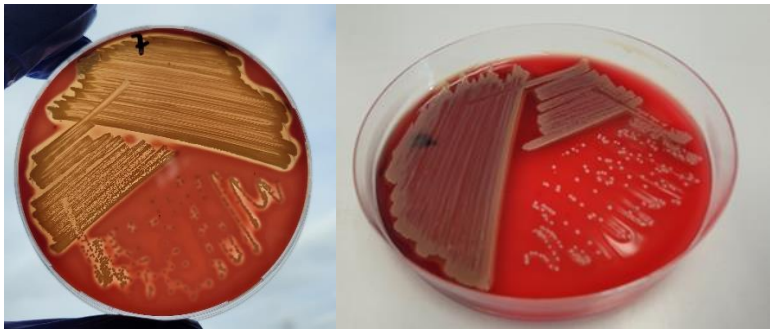

Figure 1. Colonies of *Staphylococcus aureus* on blood agar plate are gold and have double-zone haemolyses.

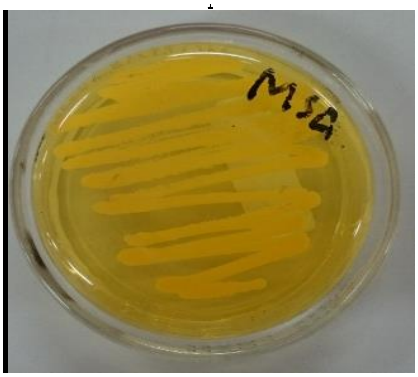

Figure 2. Colonies of *Staphylococcus aureus* are yellow on mannitol salt agar.

Figure 3. Antimicrobial susceptibility test using Kirby-Bauer disk diffusion method.

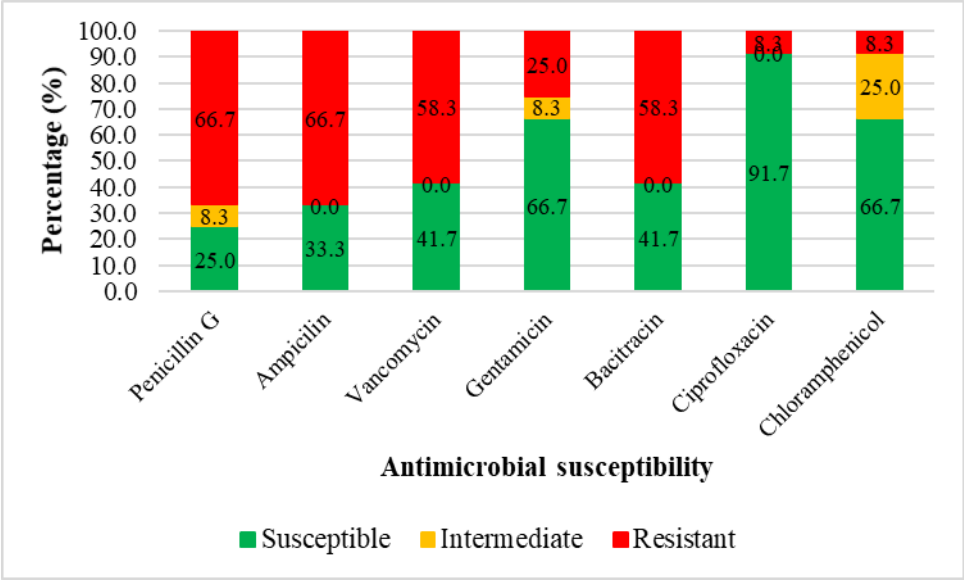

Figure 4. Antimicrobial susceptibility of *Staphylococcus aureus* (n = 12) isolated from cattle milk.