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Detection of the Gene Encoding Resistance to Ampicillin from *Staphylococcus aureus* Causing Subclinical Mastitis in Dairy Cows at Bandung District

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Abstract*— An examination of 48 samples of cow's milk was conducted, taken from three farms in the *District*s of Warnasari, Babakan Kiara, and Citawa in Bandung District. This study aimed to determine subclinical mastitis's causes and detect genes encoding bacterial resistance to ampicillin in these farms. Based on isolation and identification, PCR examinations, and phylogenetic tree examinations, the bacteria causing subclinical mastitis were identified as *Staphylococcus aureus* that genetically belongs to *Staphylococcus aureus* strains MVF-7 and DMB17 with genetic similarities of 84% and 65%, respectively, and *Staphylococcus aureus* strains SPH062R, SPH038L, and SPH029L (genetic similarity with the mecA gene of *Staphylococcus aureus* at 99.6%). Resistance to *Staphylococcus aureus* can be transmitted from livestock to farmers and the environment. The increase in antimicrobial resistance depends on the sensitivity of the compartments in humans, animals, and the environment. The level of resistance in humans is the most sensitive level when related to the parameters of the human compartment to the environment. Small-scale farmers with small livestock and land area can be a predisposing factor in increasing the spread of bacterial contamination and resistance. To stop resistance and prevent its spread, it is recommended to replace beta-lactam antibiotics such as ampicillin with other antibiotic groups that are still sensitive, such as gentamicin, oxacillin, bacitracin, and cephalosporin. Additionally, it is advised for veterinary health technicians to accurately diagnose diseases, administer correct antibiotic dosages, and select appropriate antibiotic groups that align with the cause of the disease.

***Keywords*—Mastitis; Staphylococcus aureus; resistance; mecA; ampicillin.**

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I. INTRODUCTION

Mastitis is the most common disease in dairy cattle and is recognized to have detrimental effects on cattle and livestock. Since the inception of the modern dairy farming system, producers have sought the most effective methods to minimize the occurrence of mastitis cases on their farms through animal breeding, genetic mapping, and identification of quantitative trait loci [1]. Mastitis is a disease that is a big problem throughout the world, which results in losses in the form of poor milk quality, decreased milk production, increased costs for treatment and veterinary services, high numbers of livestock being abandoned prematurely, and deaths due to the disease [2]. The high prevalence of mastitis in Indonesia can reach 83% [3], with losses that can get up to 10 million/head/year [4] in [5], making mastitis a problem. problems that must require attention to provide real and sustainable solutions. Not to mention the impact of uncontrolled use of antibiotics, which leaves another issue, namely antimicrobial residue and antimicrobial resistance.

Several bacteria have been grouped into bacteria that can cause mastitis in dairy cows. These bacteria include bacteria that cause mastitis, including *Staphylococcus aureus. Staphylococcus uberis, Staphylococcus zooepidermicus*, *Streptococcus agalactiae, S. disgalactiae, Escherichia coli, Enterobacter aerogenes* and *Pseudomonas aeruginosa*, while the causes from the fungal group are *Mycoplasma sp., Candida sp., Geotrichum sp. and Nocardia sp*. [6] and [7]. Mastitis infections caused by *Mycoplasma* are usually sporadic and without intentional intervention. This type of mastitis produces a biofilm that often does not respond to antibiotic treatment [8]. According to [9], the incidence of

mastitis in Indonesia is very high; around 80% is subclinical mastitis, while the rest are detected cases of clinical mastitis. The incidence of subclinical mastitis is very difficult to eradicate because the clinical symptoms do not appear.

To treat this, the antimicrobial groups that are widely used to treat mastitis in dairy cows include *amoxicillin*, *cloxacillin*, *penicillin*, *cephalexin*, *kanamycin*, *ceftiofur*, *tetracycline*, *streptomycin*, *neomycin*, *Kanamycin*, *lincomycin*, *rifaximin*, *novobiocin*, *sulfamethoxazole*/*trimethoprim*, *ciprofloxacin* and *enrofloxacin*. The antimicrobials most often found to have become resistant are *streptomycin*, *neomycin*, *cephalexin*, and *penicillin* [6].

Indonesia has a high level of resistance compared to other Southeast Asian countries. This is caused by inappropriate and uncontrolled use of antimicrobials in the health services sector, livestock and fisheries sector, and the ease with which people can buy antibiotics in drug stores and pharmacies without a doctor's prescription [10]. In Blitar District, the prevalence of multidrug resistance in the commercial chicken farming sector shows resistance to the beta-lactam group (79.4%), the aminoglycoside group (69%), the macrolide group (73.9%), the tetracycline group (45.8%), and the potential sulfonamide group (67%) [11]. In Pasuruan District, resistance to Staphylococcus sp. is prevalent against antibiotics penicillin 7.3%, tetracycline 7.3%, clindamycin 4.8%, and resistance to erythromycin 4.8% [12]. In the Bogor area, test results showed that 57% of *Staphylococcus aureus* were confirmed to be still susceptible to tetracycline, gentamicin, chloramphenicol, erythromycin, and trimethoprim/sulfamethoxazole, while 10.5% were resistant to methicillin [13]. In Sleman District, the percentage of resistance to antibiotics, respectively cefixime 100%; ampicillin 96%; oxytetracycline 61,5%; penicillin G38,4%; erythromycin 23%, and oxacillin 2% [14].

A. Mechanism of Staphylococcus Aureus Resistance to the Beta-lactam Group

The existence of pathogenic bacteria in the face of many different antimicrobial treatments must be able to defend themselves by protecting themselves from attack by these antibiotics. This is what is often referred to as resistance. Resistance to antimicrobial agents has become a major source of morbidity and mortality worldwide. In principle, the main resistance mechanisms are limiting drug absorption, modification of drug targets, drug inactivation, and active drug efflux. This mechanism could originate from microorganisms or be obtained from other organisms [15]. The three basic mechanisms of antimicrobial resistance are (1) enzymatic degradation of antibacterial drugs, (2) changes in bacterial proteins that are the target of antimicrobials, and (3) changes in membrane permeability to antibiotics. In the group of gram-positive bacteria, the most critical resistance mechanism to penicillin is antibiotic hydrolysis mediated by the bacterial enzyme β-lactamase. β-lactamase expression can be induced or decreased continuously through exposure to β-lactam antibiotics. Resistance to methicillin, which is always produced in gram-positive bacteria, is through changes in penicillin-binding protein (PBP) 2 by reducing the affinity of beta-lactams in the penicillin-binding protein. One of the gram-positive bacteria that has this ability is *Staphylococcus aureus* [16].

PBP is a transpeptidase involved in forming peptidoglycan in bacterial cell walls. An increase in PBP accompanied by a decrease in drug binding ability or a decrease in PBP with normal drug binding will have an impact on the amount of drug that can bind to the target in the bacteria. β-lactamase inactivates β-lactam drugs by hydrolyzing certain parts of the β-lactam ring structure, which causes the ring to open. The opening of the ring on the drug causes the drug to be unable to bind to its target PBP protein. PBP2a is a unique transpeptidase that is not inhibited well by β-lactam antibiotics. Therefore, PBP2a can continue peptidoglycan cross-linking in the face of this antibiotic challenge when other PBPs with transpeptidase activity are inhibited. The structure of PBP2a in Staphylococcus aureus bacteria is encoded by the mecA gene [16].

To find out the gene that codes for resistance to the antibiotic ampicillin in the *Staphylococcus aureus* bacteria that causes subclinical mastitis on dairy farms in Bandung Regency, it is necessary to observe milk samples taken from three areas of cattle farms that are members of the South Bandung Dairy Farm Cooperative (KPBS) in the Warnasari and Babakan Kiara in Pengalengan sub-district and Citawa *District* in Kertasari sub-district, Bandung district.

II. MATERIALS AND METHODS

A. Materials

A total of 48 milk samples were taken from three farming areas of active members of the KPBS cooperative in Bandung District. These areas are Warnasari and Babakan Kiara in Pengalengan District and Citawa *District*, Kertasari District, and Bandung District. The samples were taken during afternoon milking and after screening using the CMT test. Only positive milk samples 2 and 3 from the CMT test results will be used in the subsequent examination. Identification of milk samples was carried out in the microbiology laboratory of the Faculty of Medicine, Padjadjaran University, and to examine the genes encoding bacterial resistance, the samples were then sent to IDT Singapore via the distributor, PT Genetica Science Indonesia.

B. Method

1) Isolation and Identification: Milk samples obtained through positive CMT test 2 and 3 screening were then subjected to isolation and identification by BAP test, catalase test, coagulase test, Gram staining, and DNAse test until identified as Staphylococcus aureus group bacteria. Next, observe the Kirby Bauer bacterial growth inhibition test to see the inhibition of the growth of Staphylococcus aureus bacteria against the use of the antibiotic ampicillin.

2) Sequence Data, PCR, and Phylogenetic Analysis: The detection of coding genes was carried out by isolating sample DNA, PCR of 16s and 23s rRNA, and the mecA gene according to the protocol from ZymoBIOMIC DNA Miniprep. Information from each PCR was sequenced at Integrated Data (IDT) Technology Singapore. The sequencing results were then subjected to sequence data analysis using Blast and Creating a Phylogenetic tree using the MEGA10 program.

III. Results and Discussion

A. Results

The results obtained from the isolation, identification, and examination of the Kirby Bauer inhibition test from milk samples taken from the Warnasari and Babakan Kiara areas in Pengalengan District and the Citawa Area in Kertasari District can be seen in Fig. 1 and Table 1.

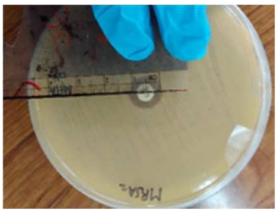

Fig. 1 Kirby Bauer Inhibition Test Results for Milk Samples (personal document)

Table 1 below shows the results of an analysis of bacterial resistance to ampicillin antibiotics through the Kirby Bauer inhibition test from milk samples taken from the Warasari, Babakan Kiara, and Citawa districts based on CLSI 2023 standards [17].

TABLE I
RESULTS OF ANALYSIS OF BACTERIAL RESISTANCE TO AMPICILLIN ANTIBIOTICS

No	Location of Sampling	Kirby Bauer Inhibition Test Results (mm)	Ampicillin Resistance Results Based on CLSI 2023
A	Warnasari (W)		
	W1	13	R
	W2	11	R
	W3	14	R
	W4	12	R
	W5	12	R
	W6	11	R
	W7	11	R
	W8	10	R
	W9	14	R
	W10	13	R
	W11	12	R
	W12	10	R
	W13	10	R
	W14	11	R
	W15	10	R
	W16	11	R
B	Babakan Kiara (B)		
	B1	10	R
	B2 (no growth)	-	-
	B3	11	R
	B4	12	R
	B5	10	R
	B6	13	R
	B7	10	R
	B8	9	R
	B9	10	R
	B10	12	R
	B11	13	R
	B12	11	R
	B13	11	R
	B14	11	R
	B15	11	R
	B16	11	R
C	Citawa (C)		
	C1	12	R
	C2	10	R
	C3	10	R
	C4	11	R
	C5	10	R
	C6	10	R
	C7	11	R
	C8	12	R
	C9	12	R
	C10	13	R
	C11	12	R
	C12	12	R
	C13	11	R
	C14 (No Growth)	-	-
	C15	11	R
	C16	11	R

Information: R: Resistant (≤28 mm) I: Intermediate (-) S: Sensitive (≥29 mm) [17]

The bacterial isolate that was detected as *Staphylococcus aureus spp* (Table 1), which was resistant to the antibiotic ampicillin, was then subjected to PCR examination to identify that the cause of mastitis in the milk samples examined was *Staphylococcus aureus* bacteria and had the gene coding for resistance to ampicillin. The results of the PCR examination can be seen in Figure 2.

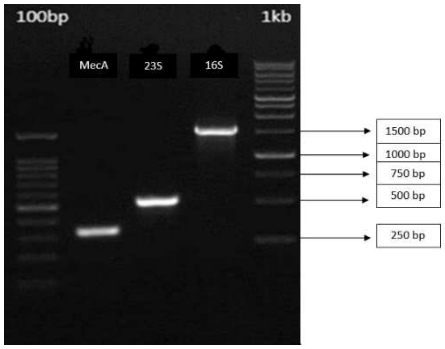

Fig. 2 Results of PCR examination of 16s and 23s mecA DNA isolates

The PCR results in Figure 2 show that each primer produced a PCR product with the expected length. Furthermore, according to the results of molecular phylogenetic analysis of the samples used in this study using the primer pair 16s (27F and 1429R) (Fig 3), it was identified as having an 84% relationship with *Staphylococcus aureus* strain MVF-7 with accession no. NR036828.1. The results of phylogenetic analysis using the 23S primer pair from this research sample showed that 65% were related to the *Staphylococcus aureus* strain DMB17 *penicillin*-binding protein (mecA) gene with accession number JN651408.1 (Fig 4). Meanwhile, examination using blast *nucleiode* analysis results showed that the mecA sequence obtained was 99.64% related to *Staphylococcus aureus* TPS3156 DNA, complete genome, and 99.6% similar to the mecA gene from *Staphylococcus aureus* strain SPH062R accession no. MK659552.1, *Staphylococcus aureus* strain SPH038L accession no. MK659551.1, and *Staphylococcus aureus* strain SPH029L accession no. MK659550.1 (Fig 3). According to the results of the phylogenetic tree examination above, the mecA gene from this research sample is related to the penicillin-binding protein 2a (mecA) gene from Stapylococcus aureus (Fig 5 and 6).

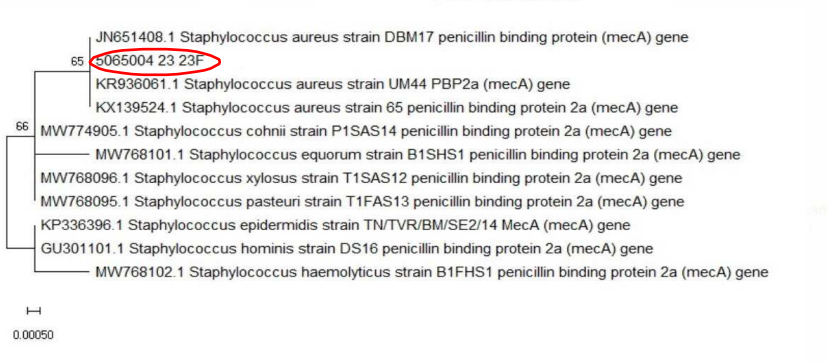

Fig. 3 The Analytic Result of Phylogenetic tree with 16s Base pairs

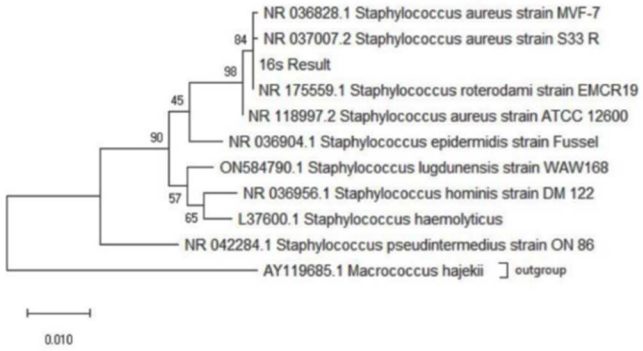

Fig. 4 The Analytic Result of Phylogenetic tree with 23S Base pairs

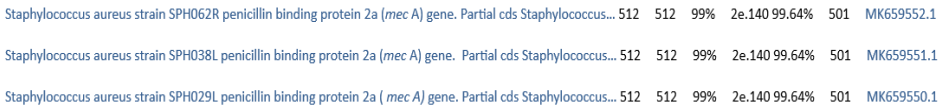

Fig. 5 The Analytic Result of Phylogenetic Sequence MecA

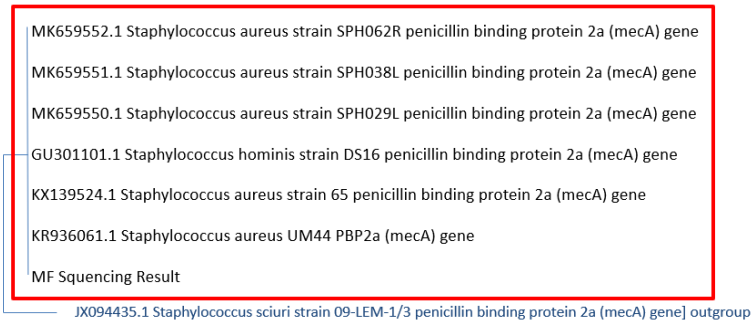

Fig. 6 Phylogenetic Families Tree of *Staphylococcus aureus* with mecA gen

B. Discussion

Based on the results of the isolation and identification of milk samples, which were then followed by DNA isolation, PCR examination, and observation of phylogenetic trees, the results showed that *Staphylococcus aureus* bacteria cause *mastitis* in dairy cows taken from the three Districts of Warnasari, Babakan Kiara, and Citawa in Bandung Regency. MVF-7 (kinship 84%), *Staphylococcus aureus* DMB17 (kinship 65%) and *Staphylococcus aureus* TPS3156, *Staphylococcus aureus* strain SPH062R, *Staphylococcus aureus* strain SPH038L and *Staphylococcus aureus* strain SPH029L (similarity to the mecA gene of *Staphylococcus aureus* 99.6%).

Data in [18] shows that *Staphylococcus aureus* MVF-7 was the first bacteria isolated from young sheep in Spain. This bacterium has a complete taxonomy as *Staphylococcus aureus Rosenbach* 1884 (Approved Lists 1980) with gram-positive morphology, coccus form, and beta hemolysis [19]. Based on its distribution, this bacterium has spread worldwide, and it is recorded that it has been isolated from water, soil, plant, and animal samples. This bacterium has been recorded as resistant to the antibiotic novobiocin and currently has resistance to ampicillin. Meanwhile, the *Staphylococcus* DMB17 bacteria, based on NCBI data [20], is a group of *Staphylococcus sp* bacteria that have not been classified (unclassified *Staphylococcus*) with taxonomic code ID: 1563697. The bacteria that cannot yet be classified are bacteria that have not yet been classified. can be differentiated based on cell structure

and metabolism or differences in cell components such as DNA, fatty acids, pigments, antigens, and quinones. This group aims to describe the diversity of bacterial species by naming and grouping organisms based on their similarities [21].

Staphylococcus aureus bacteria Strain TPS3156 with the gene encoding mecA, which codes for resistance to the antibiotic ampicillin, is presented in the NCBI 2020 GenBank data: AP023034.1 and [22] who stated that these bacteria have the gene encoding mecA, which is beta-lactam-resistant peptidoglycan from the PBP2a family, which was first reported in humans in Japan in 2020. Meanwhile, *Staphylococcus aureus* SPH062R, SPH038L, and SPH029L are bacteria that have the gene encoding mecA (penicillin-binding protein 2a partial CDS), which is listed in the NCBI gene data bank: MK659552.1; MK 659551.1 and MK 659550.1. These three strains were first reported in 2018 in nasal swabs in humans and pigs in Nigeria [23], [24].

Based on analysis data from NCBI and the first time this isolation was reported, the *Staphylococcus aureus spp* bacteria mentioned above spread from sheep and humans. Of course, the first people exposed will likely be livestock breeders, whether sheep breeders or other breeders such as cattle breeders. Meanwhile, the gene encoding mecA, which codes for resistance to the beta-lactam group of antibiotics (including ampicillin), was first reported in humans and then spread to the environment. According to [24], the increase and decrease in antimicrobial resistance depend on the sensitivity of the compartments, both humans, animals, and the environment. Resistant bacteria can enter the environment through manure on agricultural lava for vegetable and fruit crops and use in aquaculture [25]. The level of resistance in humans is the most sensitive level when it is related to the parameters of the human compartment towards the environment. This human role has a lot to do with activities such as the unwise and non-prescription sale of antibiotics, improper sanitation, and disposal of poorly metabolized antibiotics, worsening the condition [26]. The increasing transmission of resistance through the environment can have an impact on limiting antibiotic consumption in animals, both directly and indirectly related to resistance in humans themselves. In the condition of dairy farming in Pengalengan, a smallholder farmer with a small number of livestock and land relies on the rest of the yard to collect animal feed, grass, and manure in the same place. This can be a predisposing factor for increasing the spread of bacterial contamination, including the spread of bacteria that have become resistant. Likewise, the position of the cowshed is in the middle of residential areas such as the Babakan Kiara area. Small-scale community farms and traditional rearing practices tend to be less creative and slow to adopt innovation [27].

This is one of the things that causes the high resistance of *Staphylococcus aureus* bacteria to the antibiotic ampicillin in the three milk sampling areas in Bandung Regency, which reached 100%. According to [28], resistance can arise due to antibiotics not being in the correct dosage, not the proper diagnostics, and not the right bacteria that cause the disease. Antimicrobial resistance associated with Livestock Associated-Methicillin-resistant S. aureus (LA-MRSA) can reach 40% [12]. Good livestock management is one of the things that must be considered so that the development of bacterial resistance can be inhibited or even prevented. One of them is that it is recommended to replace other antibiotics that are still sensitive for treatment use in this area with antibiotics such as *gentamicin*, *oxacillin*, *bacitracin*, and the fifth-generation *cephalosporin ceftaroline* [29]. Apart from that, research on nanoparticles that have potential as antibacterials is starting to be developed because these materials provide antibacterial effects that can interfere with bacterial synthesis, including superior biocompatibility [30].

IV. Conclusion

Based on isolation, identification, and PCR examination to detect the gene encoding mecA, as well as examination of the phylogenetic tree on milk samples taken from farms in the Warnasari, Babakan Kiara, and Citawa areas of Bandung District, it was concluded that the bacteria were identified as *Staphylococcus* strains MVF-7 and DMB17 with 84% and 65% kinship similarity. Meanwhile, based on examination of the phylogenetic tree, the *Staphylococcus aureus* bacteria that have the gene encoding mecA are *Staphylococcus aureus* strains TPS3156, SPH062R, SPH038L, and SPH029L, which have a 99.6% similarity. Therefore, dairy farms in the Warnasari, Babakan Kiara, and Citawa areas of Bandung District are advised to replace the use of *beta-lactam* antibiotics such as *ampicillin* with other classes of antibiotics such as *gentamicin*, *oxacillin*, *bacitracin*, *ceftaroline*, and fifth generation *cephalosporins*. Apart from that, it is recommended that animal health technical officers carry out accurate disease diagnosis, administer antibiotic doses correctly, and select the appropriate group of antibiotics according to the cause of the disease.

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