

Determination of *mecA* Gene in *Staphylococcus spp.*, Isolate Subclinical Mastitis Ettawa Crossbred Goat Milk in Sleman Regency

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Abstract. Antibiotic treatment is one of the recommended approaches to reduce intramammary infection. Currently, antibiotic resistance is problem in the livestock treatment, especially Methicillin resistance *Staphylococcus aureus* (MRSA). The *mecA* gene has a role in MRSA coding. Therefore, the aim of the present study was to determine the *mecA* gene in *Staphylococcus spp.*, isolate subclinical mastitis Ettawa crossbred goat's milk from Sleman Regency. A total of seven *Staphylococcus spp.*, isolate subclinical mastitis Ettawa crossbred goat be composed isolate *S. aureus* (1), *S. epidermidis* (1), *S. hyicus* (2) and *S. intermedius* (3) were used. Antibiotic susceptibility against *Staphylococcus spp.*, isolate was determined by agar diffusion method using the following antibiotic Cefoxitime and Oxacillin. The *mecA* gene was detected by polymerase chain reaction (PCR). The study showed that all isolate *S. aureus*, *S. epidermidis*, *S. hyicus* and *S. intermedius* resistance Cefoxitime, whereas Oxacillin resistance occurred in two isolate *S. intermedius* and one isolate *S. hyicus*. The *mecA* gene only detected in one isolate *S. hyicus*, but none in the others isolate.

Key words: *Staphylococcus spp.*, detection, *mecA* gene, subclinical mastitis

Abstrak. Pengobatan dengan antibiotik merupakan salah satu hal yang dianjurkan untuk mengurangi infeksi ambing. Saat ini resistensi antibiotik merupakan masalah dalam pengobatan ternak, terutama Methicillin resisten *Staphylococcus aureus* (MRSA). Gen *mecA* berperan dalam menyandi terjadinya MRSA, oleh karena itu, tujuan penelitian ini adalah untuk menentukan gen *mecA* dalam *Staphylococcus spp.*, yang diisolasi dari susu kambing peranakan Ettawa mastitis subklinis dari kabupaten Sleman. Sebanyak 7 isolat *Staphylococcus spp.*, asal mastitis subklinis kambing peranakan Ettawa terdiri dari *S. aureus* (1), *S. epidermidis* (1), *S. hyicus* (2) dan *S. intermedius* (3) digunakan dalam penelitian ini. Uji kepekaan antibiotik terhadap *Staphylococcus spp.*, dengan metode agar difusi menggunakan antibiotik Cefoxitime dan Oxacillin. Gen *mecA* dideteksi dengan *polymerase chain reaction* (PCR). Berdasarkan penelitian ini menunjukkan bahwa semua isolat *S. aureus*, *S. epidermidis*, *S. hyicus* dan *S. intermedius* resisten terhadap Cefoxitime, sedangkan terhadap Oxacillin pada dua isolat *S. intermedius* dan satu isolat *S. hyicus*. Gen *mecA* hanya terdeteksi dalam satu isolat *S. hyicus* dan negatif untuk isolat yang lain.

Kata kunci: *Staphylococcus spp.*, deteksi, gen *mecA*, subclinical mastitis

Introduction

Ettawa crossbred goat subclinical mastitis is an inflammation of the mammary glands with no clinical symptoms, but somatic cell count increases commonly caused by *Staphylococcus spp.* Subclinical mastitis reduces the production of goat milk for 37- 60% (Fthenakis and Jones, 1990; Koop et al., 2010).

Antibiotic is widely administered in Ettawa crossbred goat subclinical mastitis among

farmers. However, antibiotic administration without regarding the recommended dose and duration can raise antibiotic resistance. Free injection antibiotic is very possible, especially in the farmer. Current worldwide concern is Methicillin resistance *Staphylococcus aureus* (MRSA) in which Methicillin is no longer potential against *Staphylococcus spp.*, infection in human and animal.

Methicillin resistance *Staphylococcus aureus* (MRSA) is one of *S. aureus* strain groups resistant to beta-lactam and non-beta-lactam. Groups of MRSA non beta-lactam resistance underwent change in receptor molecule or antibiotic actively pumped exit of the cell called efflux pump mechanisms (Castellanos et al., 2004). Beta-lactam antibiotic resistance occurred upon duplication in the Penicillin binding protein (PBP) such as PBP2 and PBP2a. PBP2 stopped functioning because administration of beta-lactam would compensate PBP2a, consequently, synthesis cell wall in MRSA continued (Fuda et al., 2004). PBP2a was encoded by *mecA* gene as part of the preserved genetic component called Staphylococcal cassette chromosome *mecDNA* or *mec* (SCC*mec*) (Arakere et al., 2005). The *mecA* gene and SCC*mec* belonged to MRSA and was responsible in the Methicillin mechanism resistance by degrading Penicillin ability to bind protein 2a (Duijkeren et al., 2004). Four types of SCC*mec* consisted of type one of 39 kb majorly found in 1960, type two of 52 kb in the 1980s, type three of 67 kb around the 1980s as the biggest, and type four with two subtype measuring 20.9 to 24.3 kb around 2002's (Gomes et al., 2006; Huang et al., 2006).

MRSA was reported to cause subclinical mastitis in goats. MRSA prevalence in cattle and goats subclinical mastitis was 10% and 5%, respectively (Vanderhaeghen et al., 2010; Aras et al., 2012). Methicillin resistance *S. aureus* (MRSA) was isolated from goat milk and milking worker (Stastkova et al., 2009; Aras et al., 2012). In Turki, MRSA prevalence in goat was approximately 17.5% (Turutoglu et al., 2006) while Tato et al. (2010) stated that 45% *S. aureus* caused subclinical mastitis in cow including MRSA and it had *mecA* gene. Therefore, the aim of the present study was to determine *mecA* gene in *Staphylococcus spp.*, isolate subclinical mastitis Ettawa crossbred goat's milk in Sleman Regency.

Materials and Methods

Bacterial Strains. Seven *Staphylococcus spp.*, strains isolated from Ettawa crossbred goat subclinical mastitis were used. The identification of *Staphylococcus* species was determined on the basis of Gram staining, colony morphology, catalase and biochemical test (Barrow and Feltham, 1993).

Antibiotic Susceptibility Test. Antibiotic susceptibility was determined by agar diffusion test according to Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2012) using Cefoxitime 30 µg and Oxacillin 5 µg. Each isolate *Staphylococcus spp.*, was grown in Brain Heart Infusion medium (BHI, Oxoid Ltd., Basingstoke, United Kingdom) and incubated at 37 °C for 24 hours. A total of 0.1 ml BHI medium (BHI, Oxoid Ltd., Basingstoke, United Kingdom) was dropped on the surface of Mueller Hinton Agar medium (MHA, Oxoid Ltd., Basingstoke, United Kingdom), flattened by sterile *Ose* and dried in incubator for 10 minutes. Further, MHA medium (MHA Oxoid Ltd., Basingstoke, United Kingdom) which had been inoculated with bacteria, was affixed with paper disc antibiotic and incubated at 37 °C for 24 hours. The zones of growth inhibition were evaluated as recommended by CLSI (2012).

Deoxyribonucleic Acid (DNA) Extraction. Deoxyribonucleic acid (DNA) was extracted from seven isolate of *Staphylococcus spp.*, using Qiamp tissue kit (Qiagen, Hilden, Germany) according to the manufacturer-suggested procedure. *Staphylococcus spp.*, was grown on Blood Agar Medium (BLD) (BLD; Oxoid Ltd., Basingstoke, United Kingdom) and incubated for 24 hours at 37 °C. A total of 5 colonies of *Staphylococcus spp.*, were suspended in 5 ml of BHI medium (BHI; Oxoid Ltd., Basingstoke, United Kingdom) and incubated at 37 °C for 24 hours, then centrifuged at 190 rpm for 5 minutes. Supernatant was removed and then added with 20 µl of proteinase K and 200 µl

etanol 96% into the pellet, then homogenized with vortex for 5 seconds. Sample was then moved into DNeasy Mini spin column that had been put into 2 ml collection tube. After moving, the sample was centrifuged at 8000 rpm for 1 minute, then the collection tube and DNeasy Mini spin column were put into a new 2 ml collection tube and added with 500 µl of buffer AW1. After mixing the sample with AW1, the tubes were centrifuged again at 8000 rpm for 1 minute. The collection tube was removed then DNeasy Mini spin column was placed in a new collection tube, added with 500 µl of buffer AW2 and centrifuged at 14.000 rpm for 3 minutes. The filtered liquid from collection tube was removed, then DNeasy Mini spin column then was centrifuged again at 14.000 rpm for 3 minutes to make it dry, and then the filtered liquid from collection tube was removed. DNeasy Mini spin column was relocated into micro centrifuge tubes, eluted by adding 200 µl of elution buffer AE in the central part of DNeasy Mini spin column, incubated at room temperature for 1 minute then centrifuged at 8000 rpm for 1 minute. Elution process was done twice and the collected liquid was the isolate of DNA. The DNA was kept at -20°C while DNA visualization was performed using 1% of agarose gel with Florosafe DNA Stain, and using UV transilluminator with 260 nm of wave length.

DNA Amplification. Determining the *mecA* gene was performed using Polymerase Chain Reaction (PCR) method with primer forward 5'-AAA ATC GAT AAA GTT GTT TGG C-3' and reverse 5'-AGT TCT GCA GTA CCG GAT TTG C-3' with gene size 533 base pairs (Al-Ruaily and Khalil, 2011). A total of 25 µl PCR Liquid consist of 1 µl forward primer (10 pmol), 1 µl reverse primer (10 pmol), 12,5 µl PCR mix, 1 µl template DNA, and 9,5 µl ddH₂O. These component was centrifuged for a few seconds and then moved into thermalcycler. Before running, the PCR machine was set with predenaturation temperature at 94 °C for 5

minutes, denaturation at 94 °C for 30 seconds, annealing at 50 °C for 45 seconds, elongation at 72 °C for 45 seconds, and post elongation at 72 °C for 5 minutes. These cycles were repeated 35 times. The *mecA* gene amplification product was then visualized with electrophoresis using 1% of agarose with FloroSafe DNA Stain. The DNA was visualized under UV Transluminator with 260 nm of wave length compared to the control and marker 100 base pairs (bp) of DNA Ladder.

Data Analysis. The result of antibiotic susceptibility and PCR tests were subject to descriptive analysis (Nasrullah, 1992).

Results and Discussion

Antibiotic susceptibility tests (Table 1) showed that all *Staphylococcus spp.*, isolate were resistance against antibiotic Cefoxitime and partially Oxacillin. Based on Department of Agriculture, Fisheries and Forestry in Sleman Regency, more antibiotic has been widely used in of dairy and goat farm, supported by the easy market access. It was presumably the cause caused of antibiotic resistance in goat farm in Sleman regency (Antibiotic treatment of goat in Sleman Regency, Unpublished, 2014). Contreras et al., (2000) stated that resistance caused by staphylococci produced an exopolysaccharide barrier and because of its location within microabscesses which limited the action of the drugs. In addition, antibiotic resistance can be caused by mutation gene.

Bacterial resistance against antibiotic was determined by genetic material. Bacterial resistance could derive from the environment which occurred spontaneously due to external stimuli (Todar, 2008). Antibiotic resistance was divided into two type, natural resistance and acquired resistance (Abidin, 2004); the former was when antibiotic unable to kill the bacteria in the usual or higher dose, and the latter was when bacteria previously sensitive against antibiotic became resistance (Abidin, 2004).

Table 1. Susceptibility of *Staphylococcus spp.*, isolate subclinical Ettawa crossbreed goats milk in Sleman Regency against antibiotic.

Antibiotic	MIC			Isolate						
	Inhibition Zone (mm)			A	B	C	D	E	F	G
	S	I	R							
FOX 30 µg	≥18	15–17	≤14	4 [■]	9 [■]	9 [■]	10 [■]	12 [■]	10 [■]	6 [■]
OX 5 µg	≥14	13–14	≤9	12 ^Δ	10 ^Δ	9 [■]	13 ^Δ	11 ^Δ	5 [■]	0 [■]

■ ; R: Resistance; Δ ; I: Intermediate; S: Sensitive; FOX: Cefoxitime; OX: Oxacillin; MIC: Minimal Inhibitory Concentration; A: *Staphylococcus aureus*; C, D & G: *Staphylococcus intermedius*; B & F: *Staphylococcus hyicus*; E: *Staphylococcus epidermidis*

Cefoxitime and Oxacillin are antibiotic for *Staphylococcus spp.*, producing β -lactamase enzyme. The β -lactamase enzyme causes penicillin antibiotic and other derivatives active, therefore, substituted by Cefoxitime and Oxacillin (Bywater, 1991). In some European country, Cefoxitime was still used to eliminate mastitis, especially Methicillin resistance *Staphylococcus aureus* (MRSA). Methicillin resistance *Staphylococcus aureus* (MRSA) was isolated from milk goats and milker hand (Stastkova et al., 2009; Aras et al., 2012). Prevalence of MRSA in cattle and goat with subclinical mastitis was 10%, 5% respectively (Vanderhaeghen et al., 2010; Aras et al., 2012). Occurrence of MRSA in goats in Turkey was approximately 17.5% (Turutoglu et al., 2006), while Tato et al., (2010) stated that 45% of *S. aureus* causes subclinical mastitis in cattle including MRSA strains.

Staphylococcus spp., susceptibility from goats milk against antibiotic has been tested by some previous researcher. Viridis et al., (2010) found that 25 of *S. aureus* isolated from subclinical mastitis goat milk was resistant to Kanamycin (28%), Oxytetracycline (16%), Ampicillin (12%), while 75 isolates of coagulase-negative staphylococci was resistant to Ampicillin (36%) and Kanamycin (6.7%). *Staphylococcus aureus* isolated from Ettawa crossbred goat clinical mastitis in Sleman district was resistance to Ampicillin, Erythromycin and Tetracycline (Suharto and Wahyuni, 2011). In contrast, previous study reported that *S. aureus* isolated from clinical mastitis Ettawa crossbred goat in Sleman

district was resistant to Oxytetracycline, Tetracycline, Gentamicin, Ampicillin and Erythromycin (Purnomo et al., 2006).

The result of DNA isolation is presented in Figure 1. The Figure 1 showed that DNA quality from *S. aureus*, *S. epidermidis*, *S. hyicus* and *S. intermedius* performed amplification of the *mecA* gene. This *Staphylococcus spp.*, was analyzed for *mecA* gene using polymerase chain reaction (PCR) and *S. hyicus* was positive of *mecA* gene (Figure 2).

Figure 2 showed that only one *S. hyicus* had *mecA* gene while the others were negative. Despite resistance of all phenotypes *Staphylococcus spp.*, isolate subclinical mastitis Ettawa crossbred goat against antibiotic Cefoxitime and partially Oxacillin, different genotypic character occurred such as *mecA* gene. Cefoxitin and Oxacillin are antibiotic used in staphylococci infection especially produced β -lactamase. The *mecA* gene importantly served in the resistance of MRSA by depleting penicillin ability to bind protein 2a (Duijkeren et al., 2004). The *mecA* gene was found in *Staphylococcus spp.*, such as *S. fleurettii*, *S. epidermidis*, *S. haemolyticus* and *S. xylosus* (Frey et al., 2013); pork meat (Hassler et al., 2008), and pork with exudative dermatitis (Park et al., 2013). Occasionally, phenotypic *Staphylococcus spp.*, was resistant to Methicillin, but it had no *mecA* gene. *Staphylococcus aureus* isolated from subclinical mastitis in dairy cows did not have *mecA* gene (Pinanditya, 2014). Accordingly, resistant to Methicillin did not depend on *mecA* gene existence, but also determined by others gene

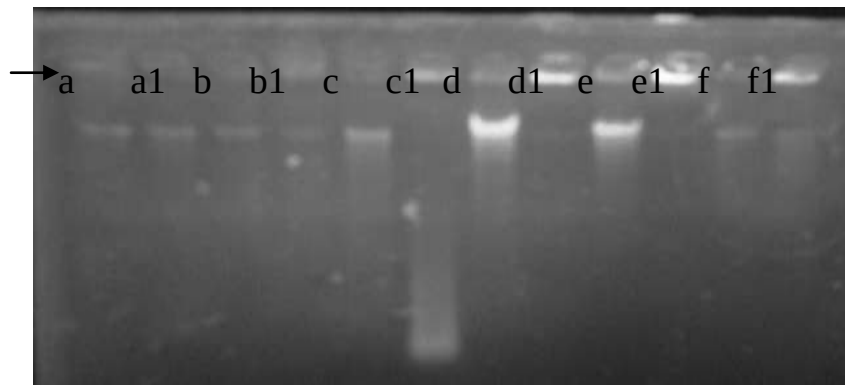


Figure 1. Isolation of DNA *S. aureus* (a & a1), *S. hyicus* (b, b1, f & f1), *S. intermedius* (c & c1) and *S. epidermidis*., after electrophoresis

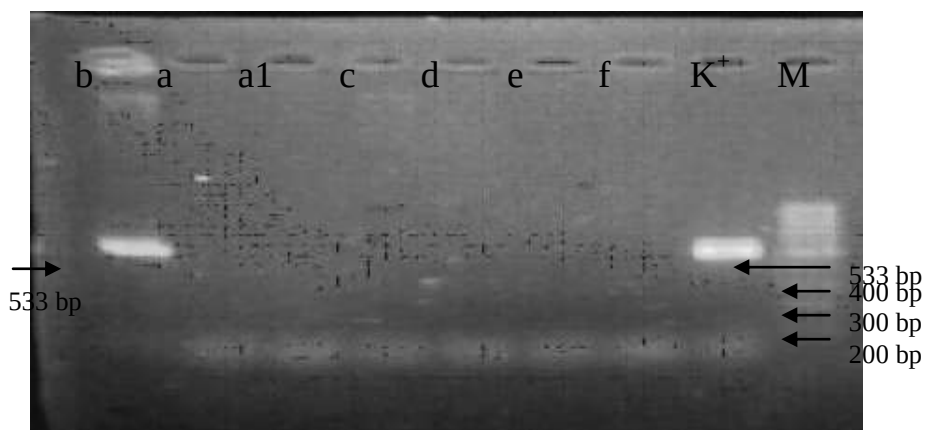


Figure 2. PCR results observed after electrophoresis show the presence specific *mecA* gene at position 533 base pairs (bp) in *S. hyicus* (b) and none in *S. aureus* (a & a1), *S. hyicus* (f) *S. intermedius* (c & d), and *S. epidermidis* (e), K⁺: positive control *mecA* gene; M: DNA Marker 100 bp

such as *Blaz Z* gene or with a particular mechanism. *Blaz Z* gene encoded the β -lactamase of penicillin by breaking β -lactam ring (Chambers, 1997).

Mechanism of antibiotic resistance had three types (Todar, 2008). First, releasing antibiotic from the cell actively or efflux pump located in the membrane cell. Second, inactivating antibiotic with the specific enzyme to eliminate antibiotic activity, and the last was degrading antibiotic with enzyme producing bacteria.

Conclusions

Staphylococcus hyicus that isolated subclinical mastitis Ettawa crossbred goat milks from Sleman regency had *mecA* gene.

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