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ORIGINATING FROM DAIRY
COW'S MILK IN SURABAYA

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Submission date: 19-Dec-2021 10:39PM (UTC+0800)

Submission ID: 1733695509

File name: JKH_Staph.pdf (623.41K)

Word count: 3580

Character count: 19459

APPLICATION OF SPA-GENE AS A MOLECULAR EPIDEMIOLOGICAL MARKER IN CASES OF *Methicillin-Resistant Staphylococcus aureus* ORIGINATING FROM DAIRY COW'S MILK IN SURABAYA

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ABSTRACT

The aim of this study was to isolate and identify the strain of *methicillin-resistant Staphylococcus aureus* (MRSA) from cow's milk in Surabaya and to determine the fragment Spa-Gene in MRSA strains. There were 50 samples of cow's milk obtained from four dairy farms. From 50 tested samples, 19 samples (38%) were positive for *Staphylococcus aureus*. Antibiotic sensitivity test using oxacillin and erythromycin showed that 6 samples were resistant to the antibiotic oxacillin and 6 samples were resistant to erythromycin. MRSA confirmation tests that were conducted on 6 samples revealed 5 positive results for the MRSA strain. Electrophoresis of Polymerase Chain Reaction (PCR) product showed that 4 out of 5 samples were positive for the presence of the Spa-Gene fragment. The research results showed that there were 3 models of Spa-Gene fragments; the first had a length of 90 bp and 140 bp, the second had a length of 140 bp, and third had a length of 90 bp. This study revealed the nature of Spa-Gene polymorphism of MRSA strains isolated from milk samples. It was concluded that the Spa-Gene can be used as a molecular epidemiological marker of the MRSA strain.

Key words: *methicillin-resistant Staphylococcus aureus* (MRSA), Spa-Gene, polymorphism, *Staphylococcus aureus*

ABSTRAK

Penelitian ini bertujuan mengisolasi dan mengidentifikasi strain *methicillin-resistant Staphylococcus aureus* (MRSA) dari susu sapi di wilayah Surabaya dan penentuan fragmen Spa-Gene pada strain MRSA. Sampel susu sapi sebanyak 50 sampel didapat dari 4 peternakan sapi perah. Hasil uji identifikasi 50 sampel didapatkan 19 sampel (38%) positif bakteri *Staphylococcus aureus*. Uji sensitivitas antibiotik menggunakan antibiotik oxacillin dan erythromycin menunjukkan 6 sampel yang resisten terhadap antibiotik oxacillin dan 6 sampel yang resisten terhadap antibiotik erythromycin. Uji konfirmasi MRSA dilakukan pada 6 sampel *Staphylococcus aureus* didapatkan 5 sampel positif strain MRSA. Hasil Elektroforesis Polymerase Chain Reaction (PCR) dari 5 sampel yang positif strain MRSA terdapat 4 sampel yang positif menunjukkan adanya band DNA hasil amplifikasi primer Spa-Gene. Hasil penelitian menunjukkan adanya 3 model gambaran fragmen Spa-Gene; pertama memiliki panjang 90 bp dan 140 bp, kedua memiliki panjang 140 bp, dan ketiga dengan panjang 90 bp. Penelitian ini mengungkapkan sifat polimorfisme Spa-Gene dari strain MRSA yang diisolasi dari sampel susu. Disimpulkan bahwa Spa-Gene dapat digunakan sebagai penanda epidemiologi molekuler dari strain MRSA.

Kata kunci: *methicillin-resistant Staphylococcus aureus* (MRSA), Spa-Gene, polimorfisme, *Staphylococcus aureus*

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a Gram positive bacterium that can live a normal human flora on the skin, as well as inside the nose and throat (Ryu *et al.* 2014). *S. aureus* causes various diseases through tissue invasion mechanisms and the production of toxins. The infection can be either local or systemic (Gladwin and Trattler, 2011). In 1941, the treatment of *S. aureus* infection was carried out by administering penicillin antibiotics, but in 1948 it was reported that *S. aureus* was resistant to penicillin. The resistance continued to grow and in 1961 researchers discovered an *S. aureus* strain that was resistant to methicillin, which became known as *methicillin-resistant Staphylococcus aureus* (MRSA) (Chambers, 1997; Giesbrecht *et al.*, 1998).

MRSA infections have become a serious problem worldwide and are currently very difficult to treat because this bacterium is resistant to all β -lactam antibiotics (Tato, 2011) and some non- β -lactam antibiotics. In the distribution pattern, MRSA is thought to have the same pattern as *S. aureus*. Several reports say that MRSA strains have been found in samples of foodstuffs from animals, such as cow's milk

and several types of cheeses in Italy (Normanno *et al.*, 2007; Kamal *et al.*, 2013).

Molecular epidemiology studies show that a number of MRSA strains are rapidly growing and capable of spreading to different regions, cities, countries, and even continents (Enright *et al.*, 2002). One factor that contributes to the spread of MRSA strains is the emergence of resistance genes other than the *mecA* gene (the gene that is resistant to all β -lactam antibiotics). For example, there are genes resistant to macrolide antibiotics, tetracycline, and aminoglycosides, which makes the infection very difficult to treat (Kevorkijan *et al.*, 2009), and also results in no clear dispersion patterns around the world (Bustamante, 2011).

The rapid development of MRSA strains is generating great interest in terms of tracking, identifying, and understanding the diversity of MRSA under a variety of circumstances. Various techniques can be used to differentiate *S. aureus* isolates, specifically MRSA. Historically, isolates can be distinguished through the phenotypic method by testing antibiotic sensitivity and genotyping methods by molecular typing. The molecular typing method can be used to classify or define the relationship between

strains isolated from a particular place and time of MRSA strains. Currently, the most widely used molecular technique for understanding MRSA diversity is via the Staphylococcus protein A (spa) (Harmsen et al., 2003; Stefani et al., 2012).

Protein A is a Staphylococcus surface protein with a molecular weight of 42 kD which is covalent in the cell wall (Schäfer and Kroppenstedt, 1990). Genes encoding protein A (Spa-Gene) are the most widely used epidemiological markers for molecular typing because they contain polymorphic units and are a good choice for identifying and differentiating *S. aureus* variability (Shakeri et al., 2010). In a study by Normanno et al. (2015), 18 genetic types of Spa-Gene were found from 31 positive MRSA samples isolated from slaughterhouses in Southern Italy. Mehndiratta et al. (2009) stated that genetic diversity is based on several factors, such as the presence of various mutations like the accumulation of point mutations, genetic rearrangement, and the acquisition or loss of chromosomal or extrachromosomal genetic elements.

Understanding the molecular epidemiology of MRSA strains in cow's milk in Surabaya is a recent and essential review. Based on the background of this problem, this research was carried out on the application of Spa-Gene as a molecular epidemiological marker in cases of MRSA originating from cow's milk collected from 4 farms in Surabaya.

MATERIALS AND METHODS

The search used cow's milk samples, Blood Agar (BA), Mannitol Salt Agar (MSA), Muller Hinton Agar (MHA), H₂O₂, rabbit blood plasma, Gram staining (Crystal violet, safranin, lugol, alcoholic setone, ChromID™ MRSA, Oxacillin and Erythromycin antibiotics, TE buffer, Spa-Gene primers, PPP Master Mix (Top-Bio, Prague), 2% agarose gel, and a 100 bp marker.

In Surabaya, 50 cow's milk samples were obtained from 4 farms located in Kaliwaron, Platuk, Wonocolo Gg 6, and Wonocolo Gg buntu. The sampling used a simple random sampling method. The milk samples were stored in an icebox for later observation. *S. aureus* was identified and isolated from the samples which then processed to antibiotic sensitivity test, MRSA confirmation test, and Polymerase Chain Reaction

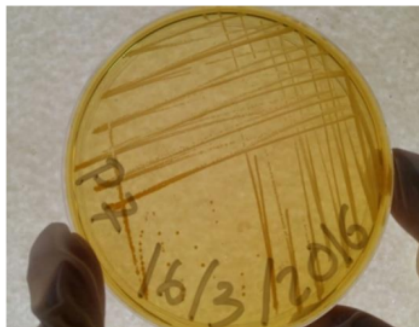


Figure 1. Isolation of *Staphylococcus* sp. on MSA media

(PCR) test to determine the fragment description of the Spa-Gene. The data from the identification results of *S. aureus*, the antibiotic sensitivity test, the MRSA confirmation test, and the PCR test were processed qualitatively and analyzed descriptively.

RESULTS AND DISCUSSION

The identification test for *S. aureus* bacteria used laboratory standards in the form of a fermentation test on MSA media, a microscopic test with Gram staining, a catalase test to differentiate *Staphylococcus* sp. with *Streptococcus* sp., a hemolysis test on BA medium, and a coagulase test which was useful for differentiating the types of *Staphylococcus* sp. This identification aimed to obtain pure isolates of *S. aureus* from cow's milk.

MSA media is one of the bases in the identification of *S. aureus* since these bacteria could ferment mannitol to form acid and change the color of the media from red to yellow. The results yielded 27 samples of the colony that were yellow and able to ferment mannitol, which is shown in Figure 1.

The identification of *Staphylococcus* sp. was followed by Gram staining. Gram staining was used to distinguish groups of Gram positive and negative bacteria, in addition to distinguish the morphology of bacteria in the form of coccus and bacillus. *Staphylococcus* sp. is shaped like a coccus and is seen clustered on a light microscope at 1000x magnification. The purple coloring indicated that the bacteria were Gram positive in Figure 2.

The next stage of identification was carried out using a catalase test. The function of the catalase test in cocci shaped bacteria is to differentiate between *Staphylococcus* sp. and *Streptococcus* sp. *Staphylococcus* sp. is known to produce the enzyme catalase which can break down hydrogen peroxide (H₂O₂) into water (H₂O) and oxygen (O₂). The catalase test was carried out on 27 samples and the results were all (100%) positive catalase. Positive catalase was indicated by gas bubbles (O₂) that are shown in Figure 3.

Gas bubbles arose because there was a reaction from the catalase enzyme which broke H₂O₂ into O₂ and H₂O. Bacteria that produced the enzyme catalase would reduce the bactericidal properties of H₂O₂ (Brooks et al., 2004). H₂O₂ is toxic to cells because it inactivated enzymes in cells. H₂O₂ was formed during

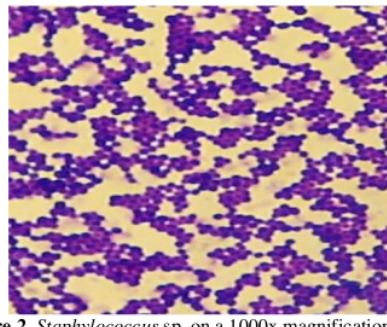


Figure 2. *Staphylococcus* sp. on a 1000x magnification light microscope

aerobic metabolism, so microorganisms that grow in an aerobic environment had to break down the toxic material (Lay, 1994).

The identification stage of *S. aureus* was followed by a hemolysis test in a BA medium. *S. aureus* would produce a β -hemolysin toxin or total hemolysis. The result was the 19 samples were β -hemolysin, marked by the presence of a bright zone around the bacterial colony on Blood agar, which indicated that bacteria can dissolve blood as shown in Figure 4.

The final identification was followed by a coagulase test. A positive coagulase test result was very important

to differentiate *S. aureus* from other *Staphylococcus*. *S. aureus* is able to produce coagulase, which is an enzyme-like protein which, when added with oxalate or citrate, can coagulate plasma. The result was the 19 samples showed positive results in the presence of plasma clots as shown in Figure 5.

Based on the identification test stages that had been carried out on 50 samples of cow's milk, 19 (38%) samples contained *S. aureus* isolates. Next, the 19 samples were tested for 2 antibiotic sensitivities. In Table 1, the number of samples taken from each farm and the number of positive samples for *S. aureus* are shown.

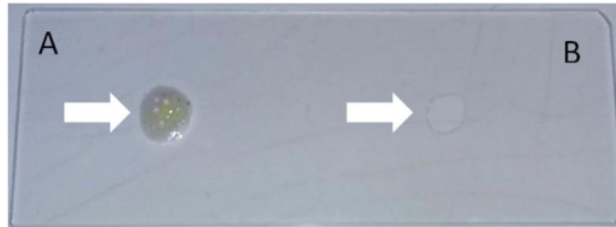


Figure 3. The catalase test results on *Staphylococcus* sp. A= Positive result; B= Negative result

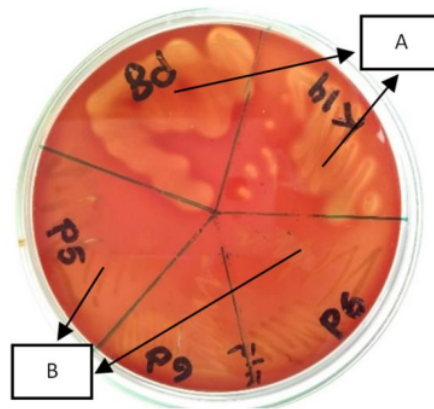


Figure 4. The *Staphylococcus aureus* hemolysis test results. A= Hemolysis; B= No hemolysis

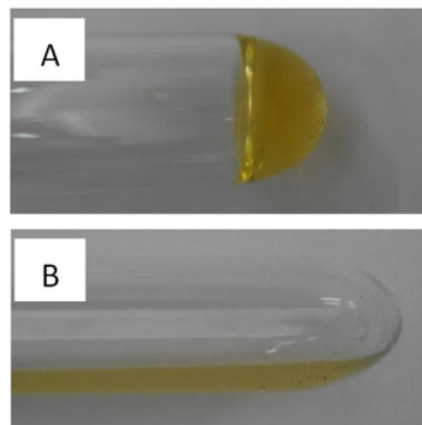


Figure 5. The positive coagulase test results *S. aureus*. A = Positive coagulase result and B = Negative coagulase result

14 After the results of the isolation and identification of *S. aureus* were obtained, then the sensitivity test was carried out to 2 antibiotics: oxacillin 5 µg and erythromycin 15 µg. The results of the sensitivity test were indicated by the presence of a zone of antibiotic resistance. The diameter of the antibiotic resistance area was measured using a ruler with units of millimeters (mm) to the bacterial growth. If there was a bacterial colony in the zone of antibiotic resistance, measurement²¹ were taken from the closest colony distance to the diameter of the inhibition zone. The results of the antibiotic sensitivity test are shown in Figure 6.

The results of 2 antibiotic sensitivity tests to 19 *S. aureus* isolates showed that 6 isolates were resistant to oxacillin and 6 isolates were resistant to erythromycin. According to Nicola et al. (1998), there are three mechanisms that lead to *S. aureus* resistance to erythromycin. The first mechanism is due to the modification of the ribosome by 23s rRNA methylase which is mediated by *ermA*, *ermB*, or *ermC*, and the second mechanism is due to the activation of the efflux.

Oxacillin is an antibiotic that is resistant to the β-lactamase (penicillinase) enzyme. β-l¹⁸am antibiotic resistance is caused by the presence of a mobile genetic element, like the *mecA* gene in SCC*mec* (Cohn and Middleton, 2010). The same species in *S. aureus* in a certain area does not necessarily show the same resistance image in other regions, it depends on the contact between the species of *Staphylococcus*²⁰ sp. and between the genus of *Staphylococcus* sp. The use of antibiotics in humans can pose a threat to both animals and products from animals by increasing the resistance of some bacteria when being transferred at interspecies and inter-genus levels (Khan, 2014).

The sample was progressed to MRSA confirmation test using ChromID™ MRSA (Biomeurix) media. ChromID™ MRSA contains a chromogenic α-glucosidase substrate and a combination of several antibiotics. The positive result for MRSA strains were indicated by isolates that were green on the media, while isolates that were not green were not MRSA strains.

MRSA confirmation test results on ChromID™ MRSA media showed that there were 5 isolates of

19 **Table 1.** The total number of samples and the number of positive samples for *S. aureus*

Farm	Kaliwaron (K)	Platuk (P)	Wonocolo Gg 6 (W)	Wonocolo Gg Buntu (WG)
The sample	15	10	13	12
Positive <i>S. aureus</i>	3	5	5	6

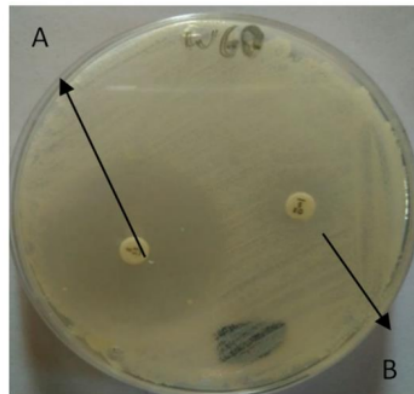


Figure 6. The antibiotic sensitivity test. A= Amoxicillin antibiotic disc; B= Erythromycin antibiotic disc

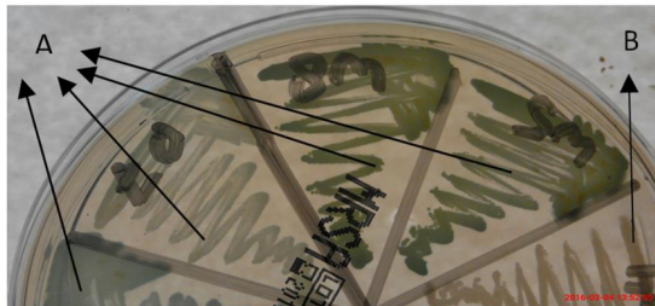


Figure 7. The MRSA confirmation test using ChromID™ MRSA media



Figure 8. The gel image of PCR amplification products. M= Marker; P= Platuk; W= Wonocolo Gg 6; WG= Wonocolo Gg Buntu

MRSA strains from 19 *S. aureus* isolates tested. The positive MRSA strain isolates were progressed to Polymerase Chain Reaction (PCR) testing, to determine the description of Spa-Gene fragments. The results obtained from the PCR is in the form of a DNA band pattern as shown in Figure 8.

The results of electrophoresis of PCR products showed that the 4 MRSA positive samples showed a DNA band, while there was 1 MRSA positive sample that did not show any DNA bands. The electrophoresis image from the PCR results showed different bands between samples from the same and different farms. The amplification results of the P8 and P7 samples from one farm were found to be different. In the P8 sample there was a length of 140 bp, while the P7 sample had a double band with a length of 90 bp and 140 bp. It was concluded that 2 samples of MRSA strains from the farm in Platuk had different clones. The samples W5 and W8 originating from Wonocolo gg 6 have the same band with a length of 90 bp and the two samples of MRSA strains were inferred from one clone. While the WG8 sample did not have any bands on the PCR results.

The results of the PCR product electrophoresis obtained 3 models of Spa-Gene fragments from 5 samples of MRSA strains. These results reveal the diversity of Spa-Gene on one farm or between farms. Spa-Gene diversity was based on band differences in PCR results. Frénay *et al.* (1994) stated that Spa-Gene is known to have polymorphic sequences that are often used to classify strains. Spa-Gene which belongs to a polymorphic sequence is called region X. Polymorphisms of region X are widely used as the basis for genotyping methods, revealing genetic differences between related strains and enabling effective epidemiological investigations. Thus, according to Schmitz *et al.* (1998) genotypic MRSA analysis is needed to determine MRSA strains that may come from one clone or different clones.

This variability in genes evolves rapidly with the times. So molecular typing is useful for routine epidemiological surveillance (Omar *et al.*, 2014). The

molecular typing system for *S. aureus* has replaced the conventional typing method. Faria *et al.* (2008) showed that 99% of *S. aureus* strains can be typed by Spa-Gene. Typing spa is a technique commonly used to classify *S. aureus* strains in clinical practice (outbreak management) and research (Votintseva *et al.*, 2014).

CONCLUSION

There are genetic differences and diversity of Spa-Gene on positive MRSA strains. The PCR results for 5 isolates consisted of P7 with a length of 90 bp and 140 bp, P8 with a length of 140 bp, W5 with a length of 90 bp, W8 with a length of 90 bp, and WG8 which do not show any DNA bands. The Spa-Gene can be used as a molecular epidemiological marker of the MRSA strain.

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