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IDENTIFICATION OF SPA GENE FOR STRAIN TYPING OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) ISOLATED FROM NASAL SWAB OF DOGS

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ABSTRACT : The purpose of this study was to identify the gene encoding protein A (spa gene) from the dog's nasal mucosa. Spa gene that encodes protein A is a virulence factor of *Staphylococcus aureus*, which has polymorphism properties and can be used for strain typing. This identification was carried out on Methicillin resistant *Staphylococcus aureus* (MRSA) and Methicillin Sensitive *Staphylococcus aureus* (MSSA). Five of the fourty five samples taken from the dog's nasal mucosa have the characteristics of *Staphylococcus aureus*; round, gram positive, fermenting mannitol, producing catalase, coagulase and acetyl methyl carbinol. The antibiotics used are penicillin, erytromycin, oxacillin, gentamycin and cefoperazone. Then do the identification of spa genes in 5 *Staphylococcus aureus* isolates using PCR techniques with specific primers.

The results of this study found a positive sample of MRSA were 3 of 5 isolates. The result of spa gene identification is the diversity of spa genes between each MRSA strain and MSSA strain. Electrophoretic picture from the results of PCR of five isolates there are three bands 220 bp, 290 bp and 600 bp, and found 2 isolates that have double band with length (290 bp and 600 bp) and (220 and 290 bp), which showed the presence of more than one allele in spa genes. The conclusion of this research is the fact that the spa gene can be used for strain typing to differentiate *S. aureus* isolates.

Key words : *Staphylococcus aureus*, MRSA, MSSA, spa Gene, dogs.

INTRODUCTION

The most pathogenic species of the genus Staphylococcus is *Staphylococcus aureus*. These bacteria often colonize the skin, mucous membranes of healthy individuals and especially the upper respiratory tract, but may not always cause clinical symptoms (Wertheim, 2005). *Staphylococcus aureus* is an opportunistic pathogen capable of attacking the respiratory tract and upper surface skin in mammals (Tulinski, 2013). This species is the most studied Staphylococcus species because it often causes infections and an increase in the prevalence of antibiotic resistant strains of *Staphylococcus aureus* (Rahmaniar, *et al*, 2020).

Diseases caused by Staphylococcus aureus are caused by the work of various virulence factors (multifactorial), therefore the regulation is complex in terms of regulating the expression of genes that encode these virulence factors (Loeffler *et al*, 2009). In humans, MRSA is recognized as a zoonotic pathogenic bacterium because of the isolation of 18% of injuries caused by dogs turned out to have the same characteristics as MRSA in dogs (Pottumarthy *et al*, 2004). Control of the spread of MRSA that can cause resistance to antibiotics and invasive infections can be done with a monitoring program (Montesinos, 2002). Molecular typing and Antibiotyping are key functions for epidemiological investigations (Omar, 2014).

Protein A is a surface protein bound to the peptidoglycan of the Staphylococcus aureus cell wall (Lee *et al*, 2004; Voyich *et al*, 2005). This protein has a role in the mechanism of bacteria infecting the host body. Among them play a role in adhesion (adhesion), colonization and destruction of cells in various body tissues. Besides the biological effects caused by slow hypersensitivity reactions and inhibit antifagocytosis opsonization. Furthermore,

protein A has the ability to bind to the Fc receptor part of immunoglobulin (Ig) in most mammalian species (Elsayed, *et al*, 2015).

The gene that encodes for protein A (spa) is the most widely used marker for molecular typing because it contains polymorphic units. Spa genes are also a good choice to be able to identify and distinguish *Staphylococcus aureus* strain variability (Kuzma *et al*, 2005; Shakeri, 2010). An understanding of the molecular epidemiology of the genotypic and phenotypic approaches of *Staphylococcus aureus* which produces various virulence factors has been reported.

The purpose of epidemiological typing is to determine the relationship between lines that were isolated from specific places and times, for example during an outbreak. At the time of outbreak, an increase in infection was found and / or resistance patterns were found to be different from previous data. Search and comparison of the lines causing the outbreak are intended to determine the type and number of strains involved, the application of appropriate therapies, restrictions on the spread of bacteria and evaluation of the success of infection control programs (Enright, 2000). Polymerase Chain Reaction (PCR) is accepted as the gold standard for detecting the characteristics of pathogenic genotypes (Effendi *et al*, 2019). Based on the background of the problem, a study was carried out on the identification of protein A coding gene (spa gene) on Staphylococcus aureus derived from dog nasal mucosal swabs using the Polymerase Chain Reaction (PCR) technique.

MATERIALS AND METHODS

Swab sampling on the dog's nasal mucosa was carried out with a cotton swab and then strreaked in stages on Mannitol Salt Agar (MSA) media then incubated at 37^0C for 24 hours. Colonies growing on Mannitol Salt agar were then examined microscopically (Hendrix and Sirois, 2007, Effendi *et al*, 2018). Biochemical test for *Staphylococcus aureus* bacteria using catalase test, coagulase test, Voges Proskauer test (VP) and hemolysis test (Quinn *et al*, 2002).

Isolated bacteria were identified, a small amount of the colon was taken with a needle loop, then planted in Mueller Hinton Broth, incubated at 37^0C for 24 hours after which bacterial growth was seen and compared turbidity suspension of bacterial suspension of 1.5x108 per millimeter according to Mac Farland standard 0.5. Antibiotic sensitivity test uses the diffusion method for Kirby Bauer. Swab evenly over the entire surface of the MHA then leave the petri dish for 5 minutes. Antibiotics are placed on the agar surface with a slight pressure using a sterile tweezers so that the disk is perfectly attached to the agar surface. Media incubation at 37^0C for 18-24 hours (Hendrix and Sirois, 2007). Disk diffusion test results show a clear or clear zone around the disk paper as an area of bacterial growth inhibition. The area of resistance is then measured using the bar. The large diameter of the inhibition area is used to determine the sensitivity of bacteria to antibiotics which are grouped into two categories namely sensitive (P), intermediate (I) and resistant (R) based on standards issued by the National Committee for Clinical Laboratory Standards (NCCLS) (CLSI, 2017) .

DNA extraction using Qiamp tissue kit (QIAGEN, Hilden Germany). The reagent for PCR consisted of 12.5 µl 2x master mix (intron), 1 µl spa primary (sense) and 1 µl primary (antisense). The primers used in this study were primers according to Akineden *et al* (2001). spa-f: 5'- CAA GCA CCA AAA GAG GAA-3' and spa-r: 3'-CAC CAG GTT TAA CGA CAT-5' with this primer in Akineden's study (2001). This can be read at length 110, 140, 170, 190, 220, 240, 270, 290 and 320 bp. 0.5 µl distilled water and 5 µl DNA template into 0.2 ml eppendorf tube. The mixture was put into a PCR machine and entered the initial denaturation stage of 94^0C 30 seconds, denaturation of 94 ^{0}C, annealing 60^0C and 72^0C extension for 1 minute with a cycle of 30 times and final extension of 72^0C for 30 seconds. The final stage, the results of the PCR are detected by electrophoresis. A total of 5 µl of PCR product from *Staphylococcus aureus* DNA was put in 2% agarose gel containing 2 µl redsafe in 20 ml of TBE 1x for the running process. After 30 minutes, the results can be visualized through ultraviolet light with a UV transluminator.

RESULTS AND DISCUSSION

Based on an analysis of 45 dog nasal mucosal swab samples, obtained 5 positive bacterial colonies fermenting mannitol on Mannitol Salt Agar medium and coccus-shaped, clustered and Gram-positive on microscopic examination. The results of identification with catalase, coagulase, Voges Proskauer (VP) tests and hemolysis tests obtained colonies with the properties of producing catalase and coagulase enzymes, producing acetyl-methyl-carbinol and forming β-hemolysis on Blood Agar media. The results showed that the positive bacterial colonies of Staphylococcus aureus numbered 5 out of 45 mucosal swabs, or about 11%.

The results of the 45 samples identification tests, can be found that there are 5 isolates that are positive for *Staphylococcus aureus*. Antibiotic sensitivity test on *Staphylococcus aureus* isolates can be shown on Table

Table 1 : The results of measurement of inhibition zones in the antibiotic sensitivity test of specimens with MHA media and incubation at 37^0C for 24 hours.

No.	Sample code	Antibiotics				
		Penicillin	**Erytromycin**	**Gentamycin**	**Cefoperazone**	**Oxacillin**
1.	Spa-1	20 (R)	22 (I)	25 (S)	35 (S)	0 (R)
2.	Spa-3	20 (R)	30 (S)	25 (S)	27 (S)	20 (S)
3.	Spa-17	40 (S)	30 (S)	27 (S)	33 (S)	15 (S)
4.	Spa-24	0 (R)	5 (R)	24 (S)	27 (S)	0 (R)
5.	Spa-35	22 (R)	0 (R)	31 (S)	35 (S)	0 (R)

Note: R: Resistant, I: Intermediate, S: Sensitive. The unit of measure is in millimeters (mm). The inhibition zone diameters in the table above in millimeters (mm) are adjusted according to CLSI (2017).

1.

Fig. 1 showed the bacteria on the MHA media are resistant to the antibiotics oxacillin, penicillin and erytromycin but are sensitive to cefoperazone and gentamycin antibiotics.

The results in this study were 3 out of 5 MRSA positive Staphylococcus aureus isolates and two which were Methicillin Sensitive *Staphylococcus aureus* (MSSA). MRSA is not only important in animal health aspects, because this bacterium is one of the zoonotic bacteria (Bhanderi and Jhala, 2011; Tyasningsih *et al*, 2019). The results of identification of spa genes by PCR technique using specific primers refer to the research of Akineden *et al* (2001) found differences in the length of spa genes in *Staphylococcus aureus* isolates, which were found to have three bands namely 220 bp, 290 bp and 600 bp (Fig. 2).

The results of studies with PCR in positive samples representing *Staphylococcus aureus* were spa 1, spa 3, spa 5, spa 14 and spa 15 samples there were different bands in the sample. spa 1 and spa 3 have a length of 290 bp, spa 14 with a length of 220 bp, spa 5 and spa 15 have two bands, spa 5 with a length of 290 bp and 600 bp and spa 15 with a length of 220 and 290 bp analyzed by PCR. This difference indicates the nature of polymorphisms possessed by the spa gene in the bacterium *Staphylococcus aureus*. Protein A in *Staphylococcus aureus* is a pathogenic factor encoded by the spa gene, showing different length variations in strains (Akineden, 2001; Shakeri, 2010).

The results of research on MRSA and MSSA strains can be found in the presence of double band from the PCR results. According to Effendi *et al* (2019), the presence of doubel band products has been explained by the presence of more than one allele in coa genes with 600, 680 and 850 bp gene products.

Virulence factors in the pathogenesis of *Staphylococcus aureus* include cell surface factors, the secreted factor. Cell surface factors include Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), Capsular polysaccharides and Staphyloxanthin. Microbial surface components recognize adhesive matrix molecules (MSCRAMMs) including Staphylococcal protein A (SpA), Fibronectin-binding proteins (FnbpA and FnbpB), Collagen-binding protein and Clumping factor proteins (ClfA and ClfB) (Elsayed *et al*, 2013).

Staphylococcal protein A (SpA) binds to IgG and helps the process of opsonization and phagocytosis. Fibronectin-binding proteins (FnbpA and FnbpB) for attaching fibronectin and plasma clots. Collagen-binding protein to help collagen tissue and cartilage. Clumping factor proteins (ClfA and ClfB) to help clot and fibrinogen (Elsayed *et al*, 2013).

Typing Methicillin Resistant *Staphylococcus aureus* (MRSA) can be performed using spa genes that encode protein A. Epidemiological data show that the spread of MRSA is one of the main objects (Harmsen, 2003). The Shopsin study found that typing using the spa gene was able to identify 27 of 29 strains (Shopsin, 1999). Spa genes are proven to function as molecular typing comparable to Pulsed Field Gel Electrophoresis (PFGE) (Kahl, 2005).

The gene that encodes protein A (spa) is the most widely used marker for molecular typing because it contains polymorphic units (Mehndiratta, 2009). The protein A coding gene is also a good choice to be able to identify and differentiate the variability of *Staphylococcus aureus* strains (Kuzma *et al*, 2005). Protein A encoded by the spa gene consists of several regions. Fc binding area consists of five replications 160 bp. Genes that encode protein A include the polymorphic Xr region of a number of variables (Lowy, 1998).

Spa-X region genes are genes that are always found in all *S. aureus* strains and show a variation of tandem repeats of around 24 bp in varying amounts (2-16 times) in the conserve region of the *S. aureus* genome (Atkins *et al*, 2008; Mehndiratta *et al*, 2009). The existence of deletions and tandem repeat in the spa gene resulted in

Fig. 1 : Antibiotic sensitivity test (a). Gentamycin antibiotic disk (b) Cefoperazone antibiotic disk (c) Erytromycin antibiotic disk (d) Oxacillin antibiotic disk (e) Penicillin antibiotic disk.

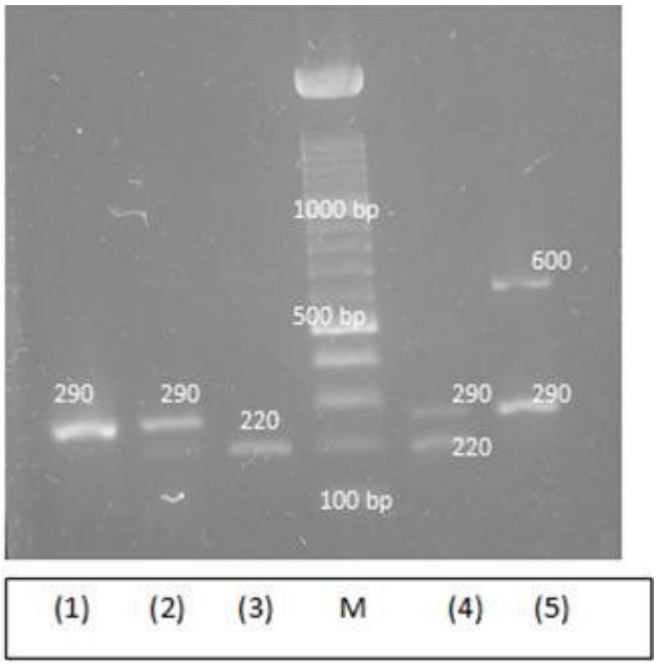

Fig. 2 : Electrophoretic DNA analysis from PCR, M: Marker; (1): spa 3 (MSSA), 290 bp; (2): spa 1 (MRSA), 290 bp; (3): spa 24 (MRSA), 220 bp; (4) spa 35 (MRSA), 220 bp and 290 bp; (5): Spa 17 (MSSA), 290 bp and 600 bp.

the emergence of polymorphism properties of spa genes which were apparently able to influence the pathogenicity of *S. aureus*. The high number of tandem repeat in the spa gene was able to increase the pathogenicity of S. aureus and was seen in *S. aureus* strains that caused epidemic cases (Montesinos *et al*, 2002).

Research on the nature of *S. aureus* spa gene polymorphisms has also been widely carried out in the world. Afrough *et al* (2013) investigated the polymorphism properties of spa and coa genes from *S. aureus* isolates from human origin showed that there are five different types of band sizes for spa genes and six different types of band sizes for coa genes via PCR. Musa *et al* (2009) also found the presence of spa gene polymorphisms for *S. aureus* isolates from abscesses in sheep and showed the presence of two different types of band sizes in the PCR test, namely 100 bp and 300 bp. Both of these studies only showed the polymorphism of the spa gene that occurred through PCR, without accompanied by tandem repeat analysis for the spa gene sequenced. Ciftci *et al* (2009) examined the polymorphism of *S. aureus* spa genes derived from cases of goat mastitis while simultaneously analyzing tandem repeat spa genes using two methods, PCR and pulsed-field gel electrophoresis (PFGE). This study produced 19 different types of spa genes from 47 *S. aureus* isolates through the PFGE test. Region X polymorphism is widely used as a basis for genotyping methods, discriminatory forces that allow recognition of small differences between genetic related strains and enable effective epidemiological investigations (Frenay *et al*, 1994).

CONCLUSION

Based on the results of the study *Staphylococcus aureus* isolates can be found from dog nasal mucosal swabs in 11%. From fourty five dog nasal mucosal swabs samples, five isolates of *Staphylococcus aureus* were found. There was amplicon diversity in the spa gene on the PCR results of *Staphylococcus aureus* derived from MRSA strains and MSSA strains from dog nasal mucosa. The results of PCR for five isolates were three bands

220 bp, 290 bp and 600 bp, and it was found that isolates that had double bands showed more than one allele in the spa gene. Spa genes can be an indicator of the determination of strains of *Staphylococcus aureus* as a marker to look for molecular epidemiological approaches.

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