Jurnal Veteriner Maret 2016
pISSN: 1411-8327; eISSN: 2477-5665
Terakreditasi Nasional SK. No. 15/XI/Dirjen Dikti/2011

Vol. 17 No. 1 : 112-118
DOI: 10.19087/jveteriner.2016.17.1.112
online pada http://ejournal.unud.ac.id/php.index/jvet.

Phenotypic and Serotypic Characterization of *Staphylococcus aureus* Strains from Subclinical Mastitis Cattle

(*KARAKTERISASI SECARA FENOTIPE DAN SEROTIPE STAPHYLOCOCCUS AUREUS YANG BERASAL DARI MASTITIS SUBKLINIK PADA SAPI*)

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ABSTRACT

Staphylococcus aureus is known as a major causative agent of mastitis in dairy cattle. In the present study, 104 isolates of *Staphylococcus* originated from subclinical mastitis cattle characterized for the phenotypic properties and the presence of Staphylococcal protein A (Spa). Some bacteria were resistances against several antibiotics were also studied, such as erythromycin, streptomycin, tetracycline, cefepime, nitrofurantoin, amikacin, chloramphenicol, and ciprofloxacin. About 78% of the isolated were moderately sensitive to nitrofurantoin, while 89% were highly resistant to cefepime and ciprofloxacin. Using the various mammals' sera, seven isolates out of 104 revealed the presence of Spa.

Key words: protein A, *Staphylococcus aureus,* subclinical mastitis, phenotyping

ABSTRAK

Staphylococcus aureus adalah penyebab utama mastitis pada sapi perah. Pada penelitian ini, sebanyak 104 isolat *Staphlylococcus yang berasal dari mastitis* subclinik pada sapi dikarakterisasi secara fenotipe dan melacak keberadaan protein-A Staphylococcus (Spa). Resistensi sejumlah bakteri terhadap juga dilakukan terhadap erythromycin, streptomycin, tetracycline, cefepime, nitrofurantoin, amikacin, chloramphenicol, dan ciprofloxacin. Sekitar 78% dari isolate tersebut bersifat sensitive sedang terhadap nitrofurantoin, sedangkan 89% sangat resisten terhadap cefepime dan ciprofloxacin. Dengan menggunakan berbagai sera mammalia, tujuh isolate terungkap menunjukkan memiliki Spa.

Kata-kata kunci: protein A, *Staphylococcus aureus,* mastitis subklinik, *phenotyping*

INTRODUCTION

Staphylococcus aureus is a major opportunistic pathogen in humans and one of the most important pathogenic *Staphylococcus* species in veterinary medicine (Peton and Loir, 2014). The emergence of antibiotic resistant strains of *S. aureus* has driven renewed interest in better defining mechanisms by which virulence factors interact with host proteins to support *S. aureus* virulence (Flick *et al*., 2013). Naturally, *S. aureus* is a commensally bacterium of the skin and mucosa of human, dairy cattle, and other warm-blooded animals. However, it is also often found to responsible for several severe infections. *S. aureus* is of particular interest due to special capacity to acquire resistance (Hoffmann *et al.,* 2015). Furthermore, circumstances such as the increasing prevalence of multidrug resistant *S. aureus* continue to press the growing international concern and need for the latest situation in farmed setting. It shows that *S. aureus* differs from other pathogens in that it has evolved as a commensally (Rasigade and Vandenesch, 2014).

In ruminants, *S. aureus* is a major causal agent of mastitis. These infections can be

relatively mild, yet serious, while life-threatening infections can be resulted from the expression of staphylococcal virulence factors that are regulated by several virulence factors (Pragman and Schlievert, 2004). *S. aureus* has capability of producing a variety of exoproteins that able to its ability to colonize the mammary gland such as hemolysins, coagulase, slime, and protein A (Coelho *et al.,* 2011). *Staphylococcus* has accessory gene regulatory D (AgrD) functioned as precursor for auto inducing peptide in a quorum-sensing system regulating virulence phenotype of the preeminent pathogen *Staphylococcus* sp. (Gonzalez *et al.,* 2014). The Agr system produces cytotoxins that act differently on the erythrocyte and *Staphylococcus* itself secretes these virulence factors including alpha, beta and delta hemolysins (Oscherwitz *et al.,* 2014). Beta and alpha hemolysins are the most important in pathogenesis of the intramamarian infections (Park *et al.,* 2004). These hemolysins can be expressed on sheep blood agar as different type of hemolysis occurred. Among above exoproteins, Staphylococcal protein A (Spa) is an important virulence factor) due to its high affinity for antibody fraction crystalizable (Fc) domains of immunoglobulin G (Fridy *et al.,* 2015) and plays a role in opsonophagocytosis during *S. aureus* infection in mammalian hosts. The Spa, a cell wall anchored protein of *S. aureus*, has the ability to interact with several host components in mammalian cell, possibly indicating a virulence factor in *S. aureus* infections (Palmqvist *et al.,* 2002). In poultry isolates, the Spa is absent due to the Immunoglobulin Y, the avian equivalent of IgG, does not bind with Spa since the IgY does not have Fc region (Lowder and Fitzgerald, 2010).

While it shares some virulence factors, *S. aureus* can also turned to be resistant to antimicrobial drugs, especially to â-lactam antibiotics (McAdam *et al.,* 2012). The antibiotic therapy of mastitis caused by *S. aureus* is generally unsuccessful due to the resistance. Furthermore, *S. aureus* has been reported to frequently cause multiple antimicrobial resistance patterns, particularly to amikacin, streptomycin, and chloramphenicol (Margariti *et al.,* 2014).

A number of immunological techniques including ELISA and Western blot have been described to detect and quantify Staphylococcal proteins (Nguyen *et al.,* 2010). The Staphylococcal protein A restriction fragment length polymorphism (RFLP) has been applied extensively to differentiate isolates of *S. aureus* with protein A (Morandi *et al.*, 2009). The methods required well-trained personal and considerably sophisticated laboratory equipment. Considering that protein A is a specific product of *S. aureus* and about 99% of the bacterial strains contain this protein (Huang, 2007), therefore we use the Spa to study the virulence of *S. aureus* isolated from subclinical mastitis cattle. In this study, the isolates were identified at the species level and the Spa was characterized by serum soft agar (SSA) technique. The isolates were also evaluated for phenotypic activities such as the presence of coagulase and hemolytic activity. In addition, the *S. aureus* strains were tested for resistance to cefepime, amikacin, streptomycin, ciprofloxacin, nitrofurantoin, and chloramphenicol.

RESEARCH METHODS

Bacterial Isolates

Isolates of 104 *Staphylococcus* isolated from bovine subclinical mastitis were the samples of the study. All isolates had been identified previously as *Staphylococcus* sp. and as a collection of Microbiology Laboratory in Veterinary Medicine Faculty, Bogor Agricultural University. *S. aureus* Cowan 1 was used as positive control while *S. epidermidis* as negative control.

Identification of *S. aureus*

Standard microbiological methods for the identification of *Staphylococcus aureus* strains were applied. Coagulase-positive staphylococci was determined used Baird-Parker Agar (Oxoid) Egg Yolk Tellurite Enrichment. The bacteria were inoculated onto the agar surfaces and incubated aerobically for 24 h at 37°C. Typical colonies of coagulase-positive staphylococci are black, shiny, and convex, surrounded by clear zones of approximately 2-5 mm. All isolates were inoculated onto manitol salt agar and incubated at 37°C. After incubation, suspect colonies were examined by Gram staining. The colonies with morphologies compatible with *Staphylococcus* spp. were transferred to Tryptic Soy Agar (TSA) (Oxoid). After growth, staphylococci were identified on the basis of colony characteristics, Gram staining, pigment production, hemolysis and the following biochemical reactions, catalase activity, coagulase test (rabbit plasma) and

manitol fermentation, finally aerobic and anaerobic utilization of glucose by FDA (2005).

Determination of Some Virulence Factors

Coagulase Test. Coagulase test is based on the ability of *S. aureus* to produce a protein product called coagulase (Mohammed and Abu-elghait, 2014). Tube coagulase (TC) technique is chosen in this study. Free coagulase activity was determined by the method described by FDA (2005). Suspect *S. aureus* colonies were transferred into small tubes containing 0.2 mL BHI broth and emulsified thoroughly. BHI culture suspensions were incubated for 24 h at 37°C. 0.5 mL coagulase plasma with Na citrate was added to the BHI culture, mixed thoroughly, incubated at 37°C and examined over 6 h for clot formation. Only firm and complete clot that stays in place when tube is inverted is considered positive for *S. aureus*.

Hemolysin Production. Alpha and beta-hemolysin were evaluated by plating strains on 5% sheep blood agar. The plates were incubated for 24 h at 37°C and then overnight at 4°C, when positive strains showed a wide zone of incomplete hemolysis with sharp edges. Non-hemolysis on 5% sheep blood agar was evaluated as gamma hemolysis (FDA 2005).

Detection of Protein A with Soft Agar (SA) and Serum Soft Agar (SSA)

The Presence of protein A was detected using soft agar technique (Wibawan *et al.,* 2009) with the addition of various mammals' serum. Bacterial suspension (1 loop) was inoculated into 10 mL of soft agar (Brain Heart Infussion (BHI) (Oxoid)+0.15%) and or into 10 mL of serum soft agar (BHI+0.15% of bacto agar+100 μL rabbit/canine/feline/sheep/chicken serum), agitated using a vortex vigorously and incubated at 37°C for 24 h. the strain containing protein A will show the changes of colony formation from diffuse in SA to compact in SSA, except SSA containing chicken serum, which was set diffuse. The negative strains will remain as diffuse colonies in SA as well as in SSA. *S. aureus* Cowan 1 is used as positive control since this bacteria known could produce high protein A. As negative control, *Staphylococcus epidermidis* is used since these bacteria cannot produce protein A

Antibiotic Resistance

Antibiotic susceptibility was determined by agar diffusion test of Muller-Hinton (Oxoid) using the following disks: cefepime (30 μg/disk), amikacin (30 μg/disk), streptomycin (10 ìg/disk), ciprofloxacin (10 ìg/disk), nitrofurantoin (300 ìg/disk) and chloramphenicol (30 ìg/disk) (Oxoid). Isolates were categorized as susceptible and resistant based upon interpretative criteria developed by Clinical and Laboratory Standards Institute (CLSI, 2007).

RESULTS AND DISCUSSION

The identification for all isolates gave primary morphological and biochemical of the *S. aureus*. From a total of 104 isolates, 8 (7.7%) were identified as *S. aureus* and 96 (92.3%) to non-*S. aureus*. Twenty-eight isolates (16.9%) have the ability to produce free coagulase.

Coagulase positive and double hemolysis are used to distinguish *S. aureus* from other Staphylococcal species. Coagulase plays as virulent factor by its ability to coaggulate blood plasma. The clotted of plasma in the coagulase test occurred caused by the converted prothrombin coagulase complex (staphylo-coagulase). Trough coagulation, staphylococci captured within a fibrin meshwork, enables this pathogen to disseminate lesions and to resist opsonophagocytic clearance by host immune cells.

Table 1. Production of different types of hemolysis by *Staphylococcus* strains

Hemolysis	104 strain of *Staphylococcus* strains
	Positive
Double(α+β)	61 (58.7%)
Beta (β)	37 (35.6%)
Gamma (γ)	6 (5.7%)

The *Staphylococcus* isolates presented different hemolysis type on sheep blood agar plates. Sixty-one (58.7%) isolates expressed double hemolysis, 37(35.6%) isolates had beta hemolysis and 6(5.7%) isolates praduced gamma hemolysis. The distribution of â-hemolysis type in this study was lower compare to previous results (Morandi *et al.,* 2009) that found hemolysin 66 of 122 *S. aureus* isolates from dairy products. Most of the *Staphylococcus* isolated derived from bovine subclinical mastitis showed double hemolysis a typical hemolisis for *S. aureus*. The distribution of double hemolysis of *Staphylococcus* isolates in this study agreed with

previous studies (Stephan *et al.,* 2001). Six out of 104 γ (5.7%) of our *Staphylococcus* isolates were *c*-hemolysis (Table 1). It is indicate that these six isolates are not belonging to *S. aureus.*

The presence of protein A can be detected in seven out of eight *S. aureus* isolates (Table 2). From the tested *S. aureus* isolates, we found *S. aureus* mutant that is showed by sample of 49, which does not express protein A. The main factor influences the release protein A to the cell wall envelope of *S. aureus* are the presence of sortase A (srtA). srtA is a transpeptidase that attaches surface proteins to the cell wall. It cleaves between Gly and Thr of LPXTG (Leu-Pro-any-Thr-Gly) motif and catalyses the formation of an amide bond between the carboxyl-group of threonine and the amino-group of the cell-wall peptidoglycan (Mazmanian *et al.,* 2001). This mechanism causes its peptidoglycan to be split and surface proteins are displayed over the staphylococcal surface. *S. aureus* lacking the srtA gene will fail to anchor and display some surface protein such as protein A. This finding can be considered that not all *S. aureus* was able to produce protein A to the surface of *S. aureus.* Moreover, the samples of 7 and 9 have less protein A than sample of 54, 58, 76, 80 and 83. However, only sample of 76 has a same pattern with Cowan 1. It indicates that sample of 76 is rich of protein A.

Staphylococcal protein A can be expressed in SSA as compact formation, which appeared globular (Figure 1). The shape related with the change of colony formation from diffuse to compact after the presence of mammals' sera in SSA (Figure 1). The compacted colony in SSA formed by *S. aureus* isolated from subclinical mastitis could partly explain the persistence and chronicity of staphylococcal subclinical mastitis. This colony is formed by growth inhibition of *S. aureus* due to IgG cover the *S. aureus* cell and this mechanism resulted the colony looks globular or compact. This form showed mechanism used by *S. aureus* to evade host defenses. It can be considered that protein A as virulence factor allows *S. aureus* to adhere of host cells and host tissues, invade of host cells, spread and manipulate of the immune response.

Figure 1. Compact colony (right), Diffuse colony (left) in Serum Soft Agar (SSA).

Table 2. Detection of protein A with SSA

Isolates	Without serum	Serum				
		Cat	Dog	Sheep	Rabbit	Chicken
(7)	D	D	D	C	D	D
(9)	D	D	C	D	D	D
(49)	D	D	D	D	D	D
(54)	D	C	C	C	D	D
(58)	D	C	C	C	D	D
(76)	D	C	C	C	C	D
(80)	D	C	C	C	D	D
(83)	D	D	C	C	C	D
Cowan 1	D	C	C	C	C	D
S. epidermidis	D	D	D	D	D	D

C (Compact), D (Diffuse), SSA (Serum Soft Agar)

Table 3. Susceptibility of *Staphylococcus aureus* to antibiotics

S. aureus	Antibiotics					
	Cefepime	Amikacin	Streptomycin	Ciprofloxacin	Nitrofurantoin	Chloramphenicol
(7)	R	R	R	R	I	S
(9)	R	R	S	R	S	I
(49)	R	I	R	R	R	S
(54)	R	I	I	R	S	S
(58)	R	I	S	R	S	R
(76)	R	R	I	I	S	S
(80)	R	I	S	R	S	I
(83)	R	I	S	R	S	R
Cowan 1	I	R	I	R	S	R
S%	-	-	45	-	78	45
I%	11	56	33	11	11	22
R%	89	44	22	89	11	33

S (Sensitive), I (Intermediate), R (Resistant)

However, the gene-encoded protein A, Spa, has host specificity and Spa is absent in poultry (Lowder *et al.,* 2009). It has been showed from this study that *S. aureus* tested in soft agar containing chicken sera cannot form compact colony (Figure 1). As mentioned previously, Immunoglobulin Y does not bind Protein A (Lowder and Fitzgerald, 2010), which this trait can show protein A only can bind to Fc region of immunoglobulin G and plays a role in opsonophagocytosis only during *S. aureus* infection in mammalian hosts.

In this study, isolates tested showed different results in each animal's serum (Table 2) that furthermore this distribution of colony form indicates the species-differences of IgG ability to bind protein A. In this study, serum of rabbit showed poor affinity to the cell while it is surprising given the rabbit serum is often used in SSA test (Djannatun 2002). Meanwhile, in our study, the canine serum and sheep serum gave the best affinity in SSA test. Therefore, the canine and sheep serum may be also advisable for further SSA test.

In this study, all of the nine *S. aureus* strains studied were tested for resistance to antibiotics. The antibiotics selected for the study regarded as the most commonly used antibiotic in the medical and veterinary fields. The results of the sensitivity or resistance of *S. aureus* were presented in Table 3. The results support the possibility of the low efficacy of antibiotic treatment for mastitis caused by staphylococci is considered multiple resistant. The results of antibiotic sensitivity test (Table 2) indicated that the pathogen, *S. aureus* showed resistant to cefepime (89%), ciprofloxacin (89%), and amikacin (44%) in a declining order. Our result was then contrary to the previous study by (Patnaik *et al*., 2014) who had observed that *S. aureus* were sensitive to ciprofloxacin (100%) and amikacin (85.72%), while Margariti *et al.* (2014) reported that *S. aureus* was sensitive to amikacin (20%). However, in the other hand, *S. aureus* was moderately sensitive to nitrofurantoin (78%) and low sensitive to streptomycin (45%) and chloramphenicol (45%). Therefore, we found that most of *S. aureus* isolates were susceptible to nitrofurantoin. This may be due to that nitrofurantoin is not used routinely for the treatment of bacterial disease in domestic ruminants in Indonesia. On the other hand, our staphylococcal isolates showed high level of resistance particularly to cefepime and ciprofloxacin, which are commonly used in treatment for bacterial infections. This high rate of resistance to the particular antibiotics in our staphylococcal collection is likely due to selective pressure from misuse (Adwan, 2006), or exacerbated by frequent usage of intra mammary infusions (Kateete *et al.,* 2013). If cefepime and ciprofloxacin were used indiscriminately, it probably will further complicate mastitis if the causative agent happens to be resistant to these antimicrobials (Ranjan *et al.,* 2010). Furthermore, the resistance of these antibiotics

has significance to public health since the transmission of resistant bacteria to the environment or dairy products could cause failure antibiotics treatment in human.

CONCLUSION

Our results confirm the wide variety of phenotype and serotype diversity of *S. aureus* from subclinical mastitis cases. Also, our study revealed that not all *S. aureus* strain could express protein A on the surface of these bacteria. SSA observation conducted one isolate revealed protein A that gave same result compare to *S. aureus* Cowan 1. *S. aureus* strains isolated from subclinical mastitis of cows has presented resistance to cefepime, ciprofloxacin, and amikacin.

SUGGESTION

Molecular assay is needed to detect the presence of SaP gene in sample showed negative result in SSA assay.

ACKNOWLEDGEMENTS

This work was funded by Dikti, research progress of PMDSU (Program Menuju Doktor Sarjana Unggulan). The authors are grateful to Agus Sumantri, Bogor Agricultural University, Bogor, Indonesia for providing necessary facilities to carry out this original research work.

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