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Genetic Determination and Clonal Relationships of *Staphylococcus aureus* Isolated from Dairy Cows in Baturraden, Central Java, Indonesia

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Abstract

Staphylococcus aureus is a relatively clonal microorganism that causes subclinical and clinical mastitis. Cases of mastitis in cows at Baturraden are leading to significant and ongoing problems due to reduced production and lower milk quality. This study was designed to identify which of selected virulence determinant genes of *S. aureus* are involved in the Baturraden infection, and to determine the clonal relationship among these isolates. Seventeen isolates were identified as *S. aureus* based on their biochemical properties and species specificity for 23S rRNA and *nuc* genes. *S. aureus* isolates were genotypically characterized for the selected virulence determinants: *coa*, *clf*A, *fnb*A, *fnb*B, *cap*5, *spa* IgG and *spa X- region* genes. Clonal relationship analysis among isolates was carried out using AFLP and results compared with previously confirmed relationships between selected *S. aureus* isolated from other regions. The results show that eight isolates contain all the genes, but six isolates lack *fnb*B and two isolates lack *cap*5 genes. AFLP analysis showed that all isolates of *S. aureus* originating from cows in Baturraden belong to one cluster. This study provides additional knowledge about *S. aureus* infection in Baturraden cows, including the number of virulence determinant genes that may play a role in pathogenicity.

Keywords: *Staphylococcus aureus*, virulence determinants, dairy cows, PCR, AFLP

Introduction

Staphylococcus aureus (*S. aureus*) is a relatively clonal microorganism that causes subclinical and clinical mastitis (Melles *et al*. 2009; Salasia *et al*., 2011). This disease causes significant economic losses in dairy farms due to decrease in milk production, shorter lactation times and increased costs for treatment and stock replacement (Halasa *et al*., 2007; Nielsen, 2009). *S. aureus* is also known as an important food-borne pathogen which has the ability to produce various virulence factors and a wide range of extracellular proteins that contribute to the pathogenicity of the organism (Nashev *et al*., 2004).

Cases of subclinical and clinical mastitis of cows are occuring at Baturraden, one of the national dairy cow centres in Indonesia, causing significant and ongoing problems due to decreased production and lower quality of milk (Agus, 1991). *S. aureus* is recognized as a major pathogen causing subclinical and clinical intramammary infections in dairy cows in Indonesia (Abrar *et al*., 2012). Therefore, this site provides an excellent opportunity to investigate the characteristics of Indonesian strains of *S. aureus*.

Tato (2012) has proposed that, in order to develop strategies to control *S. aureus* infection from various countries or areas, we need the ability to map the virulence

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determinants of bacteria, as well as the capability of the bacteria to survive the heavy pressure of various antibiotics, geographical conditions, and adaptation to environments. Salasia *et al*. (2011) reported that molecular typing of *S. aureus* and determining the clonal relationship between isolates has proved useful for epidemiological studies. Therefore, mapping the virulence determinant genes and clonal relationship patterns of *S. aureus* isolates from Baturraden could be important in trying to address the problems there.

The present study was designed to identify which of selected virulence determinant genes of *S. aureus* act in the pathogenicity of the *S. aureus* infection in Baturraden, and to determine the clonal relationship among these isolates. The results may contribute not only to knowledge of *S. aureus* in specific locations, but also to potential control strategies for staphylococcal mastitis in the future.

Materials and Methods

Milk samples and bacterial isolation

Milk samples were collected aseptically by hand milking into sterile 15 ml tubes from seventeen dairy cows from Balai Besar Pembibitan Ternak Unggul (BBPTU) Baturraden, Central Java, Indonesia. Milk samples were placed in an ice box for transport to the Faculty of Veterinary Medicine, Gadjah Mada University, for isolation of strains. The strains were identified as *S. aureus* by their properties in mannitol salt agar, Gram staining, and biochemical assays, as described by Ariyanti *et al*. (2011) and Salasia *et al*. (2011). The strains were then identified genotypically by PCR amplification of the 23S rRNA and thermonuclease *nuc* genes (Salasia *et al*., 2011). The reaction mixture (30 µL) for PCR contained 1 µL of 10 pmol of the forward and reverse primers (Table 1; Invitrogen, USA), 14 µL PCR mix (Roche, Germany) containing Taq DNA polymerase, $MgCl_2$, and dNTPs, 5 µL of DNA template, and 9 µL molecular grade water. The DNA of the isolates was prepared with the DNeasy blood and tissue kit (Qiagen, Germany) following the manufacturer's directions. The amplification of the genes was carried out with a thermal cycler (Mastercycler; Eppendorf, Germany); the thermal cycler programs are shown in Table 1.

Isolation of DNA and purification

Chrosomal DNA from *S. aureus* was isolated by a DNeasy blood and tissue kit (Qiagen, Germany) according to the manufacturer's protocol. The bacterial strains were cultivated on blood agar (Oxoid, Germany) containing 5% defibrinated sheep blood for 24 h at 37°C (Salasia *et al*., 2004). *S. aureus* colonies (5-10) were suspended with 180 µL TE buffer (10 mM Tris-HCl and 1 mM EDTA [pH 8]) containing 5 µL lysostaphin (1.8 U/µL; Sigma, USA) in 2-mL microfuge tubes. The suspension was incubated for 1 h at 37°C, and 25 µL of proteinase K (14.8 mg/mL; Sigma, USA) and 200 µL of AL buffer (containing reagents AL1 and AL2 from the kit; Qiagen, Germany) was then added. The suspensions were incubated for 30 min at 56°C, and then for 10 min at 95°C before being spun at 6,000 × g for a few seconds. Ethanol (420 µL) was added to each sample and the mixture placed in a spin DNeasy column. After centrifugation at 6,000 × g for 1 min, the spin columns were placed in a clean collection tube and the sample was washed twice with 500 µL of AW buffer (Qiagen, Germany). After the second wash and centrifugation at 6,000 × g for 3 min, the DNeasy spin columns were placed in a clean 2-mL microfuge tube, and the DNA was eluted twice with 200 µL and 100 µL of AE buffer (Qiagen, Germany). DNA was stored at -20°C (Salasia *et al*. (2011).

Genotype characterization

A PCR method was used to identify the genetic determinants of selected virulence factors. The oligonucleotide primers used amplified the virulence genes encoded by *coa*, *clf*A, *fnb*A, *fnb*B, *cap*5, *spa* IgG and *spa*

Table 1. Oligonucleotide primers and PCR programs used to amplify the genes encoding 23S rRNA, thermonuclease and selected staphylococcal virulence determinant genes identified from literature

Genes	Sequence	Program*	Refference
23S rRNA	5′ACG GAG TTA CAA AGG ACG AC 3′ 3′AGC TCA GCC TTA ACG AGT AC 5′	1	Straub *et al.,* 1999
nuc	5′GCG ATT GAT GGT GAT ACG GTT 3′ 3′ACG CAA GCC TTG ACG AAC TAA AGC 5′	2	Salasia *et al.,* 2011
*clf*A	5′GGC TTC AGT GCT TGT AGG 3′TTT TCA GGG TCA ATA TAA GC 5′	3	Salasia *et al.,* 2011
*co*a	5′ATA GAG ATG CTG GTA CAG G 3′ 3′ GCT TCC GAT TGT TCG ATG C 5′	4	Salasia *et al.,* 2011
*fnb*A	5′ GCG GAG ATC AAA GAC AA 3′ 3′ CCA TCT ATA GCT GTG TGG 5′	5	Salasia *et al.,* 2004
*fnb*B	5′ GGA GAA GGA ATT AAG GCG 3′ 3′ GCC GTC GCC TTG AGC GT 5′	5	Salasia *et al.,* 2004
*cap*5	5′ATG ACG ATG AGG ATA GCG 3′ 3′ CTC GGA TAA CAC CTG TTG C 5′	6	Salasia *et al.,* 2011
spa IgG	5′ CAC CTG CTG CAA ATG CTG CG 3′ 3′ GGC TTG TTG TTG TCT TCC TC 5′	7	Salasia *et al.,* 2004
spa X-region	5′ CAA GCA CCA AAA GAG GAA 3′ 3′ CAC CAG GTT TAA CGA CAT 5′	8	Salasia *et al.,* 2004

*1 : 30 cycles of 94^{0}C for 120 sec, 64^{0}C for 40 sec, 72^{0}C for 75 sec; 2 : 37 cycles of 94^{0}C for 60 sec, 55^{0}C for 30 sec, 72^{0}C for 30 sec; 3 : 35 cycles of 94^{0}C for 60 sec, 57^{0}C for 60 sec, 72^{0}C for 60 sec; 4 : 30 cycles of 94^{0}C for 60 sec, 58^{0}C for 60 sec, 72^{0}C for 60 sec; 5 : 30 cycles of 94^{o}C for 60 sec, 53.7^{o}C for 45 sec, 72^{0}C for 75 sec; 6 : 30 cycles of 94^{o}C for 60 sec, 54^{o}C for 45 sec, 72^{0}C for 60 sec; 7 : 33 cycles of 94^{o}C for 60 sec, 59^{o}C for 45 sec, 72^{0}C for 60 sec; 8 : 33 cycles of 94^{o}C for 60 sec, 53^{o}C for 45 sec, 72^{0}C for 30 sec.

X-region genes. The sequences of the primers and PCR conditions are shown in Table 1.

Amplified Fragment Length Polymorphism (AFLP) analysis

The DNA of the isolates was prepared with the DNeasy blood and tissue kit (Qiagen, Germany) as per by the manufacturer's protocol. An AFLP analysis was performed according to Boerema *et al*. (2006) and Salasia *et al*. (2011). Genomic DNA (5 µL) was digested overnight (16 h) at 37^{o}C with 10 U of *Hin*dIII (Invitrogen, USA) and 5 mM spermidine trihydrochloride (Sigma, USA) added to a final volume of 20 µL. The 5 µL of digested DNA was added to a ligation reaction containing 0.2 µg of each adapter oligonucleotide (ADH-1 ACG GTA TGC GAC AG and ADH-2 AGC TCT GTC GCA TAC CGT GAG) (Invitrogen, USA) and 1 U of T4 DNA ligase (Invitrogen, USA) in a final volume of 20 µL, and incubated for 4 h at room temperature (approximately 20^{o}C). The ligated DNA samples were heated for 10 min at 80^{o}C to inactivate the T4 ligase and then diluted 1 : 5 in sterile water. PCR reactions were performed in a total volume of 50 µL containing 2.5 µL of template DNA, 200 µM of dNTPs (Roche, Germany), 1 µL of HI-X primer (GGT ATG CGA CAG AGC TTX, where X = A, T, G or C; 100 pmol/µL; Invitrogen, USA), and 1 µL (5 U) of *Taq* DNA polymerase (Roche, Germany) in 1 × PCR buffer provided by the manufacturer (Roche, Germany). Each HI-X primer was used in four separate PCR reactions. Amplification was performed in a thermal cycler (Eppendorf, Germany). After initial denaturation for 4 min at 94^{o}C, target gene fragments were amplified for 33 cycles. Each cycle consisted of a denaturation step for 1 min at 94^{o}C, an annealing step for 1 min at 60^{o}C, and an extension for 2.5 min at 72^{o}C. An additional base pair was incorporated into each adapter oligonucleotide in the restriction site in order to eliminate it after

the ligation reaction. The PCR products were separated by gel electrophoresis in a 1.5% (w/v) agarose gel (Roth, Germany) in 0.5 × TBE buffer (containing a mixture of Tris base, boric acid and EDTA). A 1-kb Plus DNA ladder (Invitrogen, USA) was used as a size marker. The resulting bands were visualized using ethidium bromide staining under UV transillumination and the gel images were evaluated visually. AFLP patterns were analyzed using NTSYSpc 2.1 software (Applied Biostatistic, New York). Dendrograms were obtained with the average linkage method (unweighted pair group average method, UGPMA) following Boerema *et al*. (2006) and Salasia *et al*. (2011).

Results and Discussion

Seventeen isolates were identified as *S. aureus* (Table 2). All 17 cultures were Gram positive and positive for catalase, coagulase, VP test, and clumping factor reaction on microscope slides (data not shown). Eight isolates contained all the *coa*, *nuc*, *clf*A, *fnb*A, *fnb*B, *cap*5, *spa* IgG, and *spa* X-region genes of various sizes, but six isolates lacked *fnb*B and two isolates lacked *cap*5 genes (Table 2).

The proteins encoded by *coa*, *fnb*A, *clf*A and *fnb*B, *spa* IgG and the *spa* X-region contribute to the avoidance mechanism of *S. aureus* against the host immune system. These genes, except the *coa* gene, encode adhesin proteins that can bind to fibrinogen, elastin and fibronectin, mainly on the surface of epithelial cells. This binding is an important stage in the process of colonization and infection through the attachment of bacteria to host cells and extracellular matrix. The ability of *S. aureus* to bind fibrinogen could inhibit the process of opsonisation of *S. aureus* (Pratomo *et al*., 2012).

S. aureus produces an enzyme called coagulase encoded by the *coa* gene. This is a polypeptide that binds to and activates prothrombin, thereby converting fibrinogen to fibrin and promoting clotting of plasma or blood and leading to the formation of abscesses (Cheng *et al*., 2010). Seventeen isolates in this study contain this gene, with polymorphism in size; two isolates have sizes of 600 bp and the others are 900 bp (Table 2). da Silva and da Silva (2005) and Saei *et al*. (2009) reported that the *coa* gene can be used as an epidemiological marker for typing *S. aureus* isolates.

Amplification of *spa* IgG and *spa* X-region genes in the present study showed the same amplicon sizes as the previous study by Salasia *et al*. (2004). However, the current study showed the polymorphism bands of the *spa* X-region gene for two isolates with sizes of 100 bp and for the other 15 isolates 200 bp (Table 2). The *spa*-X region exists in all strains of *S. aureus*, where X-region indicates tandem repeats with sizes ranging from 24-26 bp in conserved areas in the genome of *S. aureus*. Protein A, encoded by *spa* IgG and *spa* X-region, acts to bind the Fc region of IgG, which has an important role in the phagocytosis mechanism through the classical pathway of complement activation (Atkins *et al*., 2008).

Capsular polysaccharides of *S. aureus* also play an important role in the pathogenesis (Salasia *et al*., 2004), causing invasive disease (O'Riordan and Lee, 2004) by protecting the bacteria from phagocytosis of leukocytes and facilitating attachment to host epitel and endotel cells. The study shows that 15 *S. aureus* isolates (88%) have a *cap*5 gene. This result agrees with the previous finding of Salasia *et al*. (2011), who reported that most *S. aureus* isolated from cows have *cap*5 genes.

Amplification of the 23S rRNA, *nuc*, *clf*A, *fnb*A, *fnb*B, *cap*5 and *spa* IgG genes produced amplicons with approximate sizes of 1250, 1050, 300, 1300, 900, 880 and 900 bp, respectively. The *coa* gene and the *spa* X-region showed polymorphism bands. The size of the *coa* gene in two isolates was 600 bp and in the other 15 isolates was 900 bp; similarly the *spa* X-region gene showed two sizes: 100 bp in the same isolates as they had smaller size for the *coa* gene and 200 bp in the other isolates (Figure 1 and Table 2).

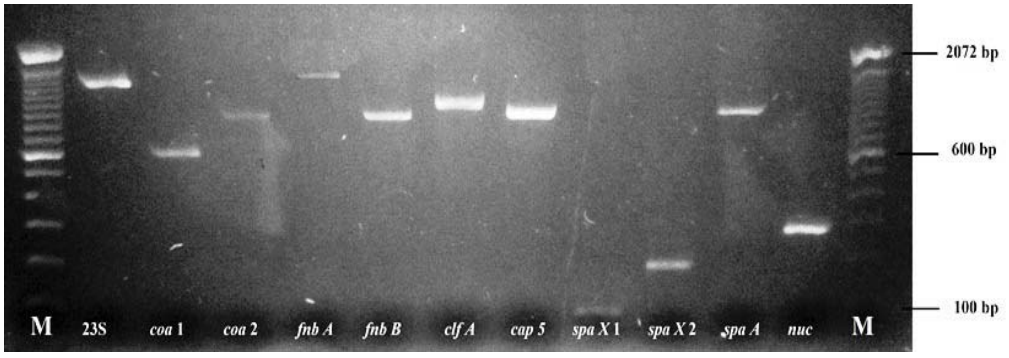

Figure 1. Agarose gel electrophoresis image of virulence determinant genes of *S. aureus*. M = 100 bp DNA marker (Invitrogen).

Table 2. Genotypic characterization of 17 *S. aureus* isolates from dairy cows in Baturraden, Central Java, Indonesia

No	Isolate code	23S r RNA (1250bp)	*coa* (900bp)	*nuc* (300bp)	*clf*A (1050bp)	*fnb*A (1300bp)	*fnb*B (900bp)	*cap*5 (880bp)	*spa* IgG (900bp)	*spa X region* (200bp)
1	BR03b	+	2	+	+	+	+	+	+	+
2	BR39a	+	2	+	+	+	+	+	+	+
3	BR13a	+	2	+	+	+	+	+	+	+
4	BR00b	+	2	+	+	+	+	+	+	+
5	BR90c	+	2	+	+	+	+	+	+	+
6	BR02a	+	2	+	+	+	+	+	+	+
7	BR67c	+	2	+	+	+	+	+	+	+
8	BR38d	+	2	+	+	+	+	+	+	+
9	BR30d	+	2	+	+	+	-	+	+	+
10	BR40c	+	2	+	+	+	-	+	+	+
11	BR82a	+	2	+	+	+	-	+	+	+
12	BR75a	+	2	+	+	+	-	+	+	+
13	BR78a	+	2	+	+	+	-	+	+	+
14	BR74b	+	2	+	+	+	-	+	+	+
15	BR49b	+	1[a]	+	+	+	+	-	+	+[b]
16	BR49c	+	1	+	+	+	+	-	+	+[b]

[a]gene size: *coa*: 1 (600 bp), 2 (900 bp), [b]*spa X region*: (100bp).

AFLP was performed using basic PCR combined with a restriction enzyme and a DNA ligase to cut the genome at specific sites and ligate it with the appropriate adapter using a restriction enzyme (Janssen *et al*., 1996). This study used the primer HI-G (ATG GGT AGC TTG CGA CAG) as used by previous investigations (Boerema *et al*., 2006; Salasia *et al*., 2011). To compare the results of the AFLP analysis, we used 10 *S. aureus* isolates from dairy cows in Yogyakarta (SU2, SU10, SU24, SU25 SU16, and SU28), Solo (SU34 and SU39) and Boyolali (BY5 and BY7) that had been previously generated by Salasia *et al.* (2011). These data were used to unravel the relationship between isolates from Baturraden and those from the other regions (Figure 2).

The dendrogram in Figure 2 shows that *S. aureus* isolated from Baturraden group in one cluster, which has a similarity coefficient of 80%. These isolates had a distant relationship with the ones collected from Yogyakarta and Solo, with a similarity coefficient of 62%, but the Baturraden isolates had a closer relationship with those from Boyolali, with a similarity coefficient of 78%.

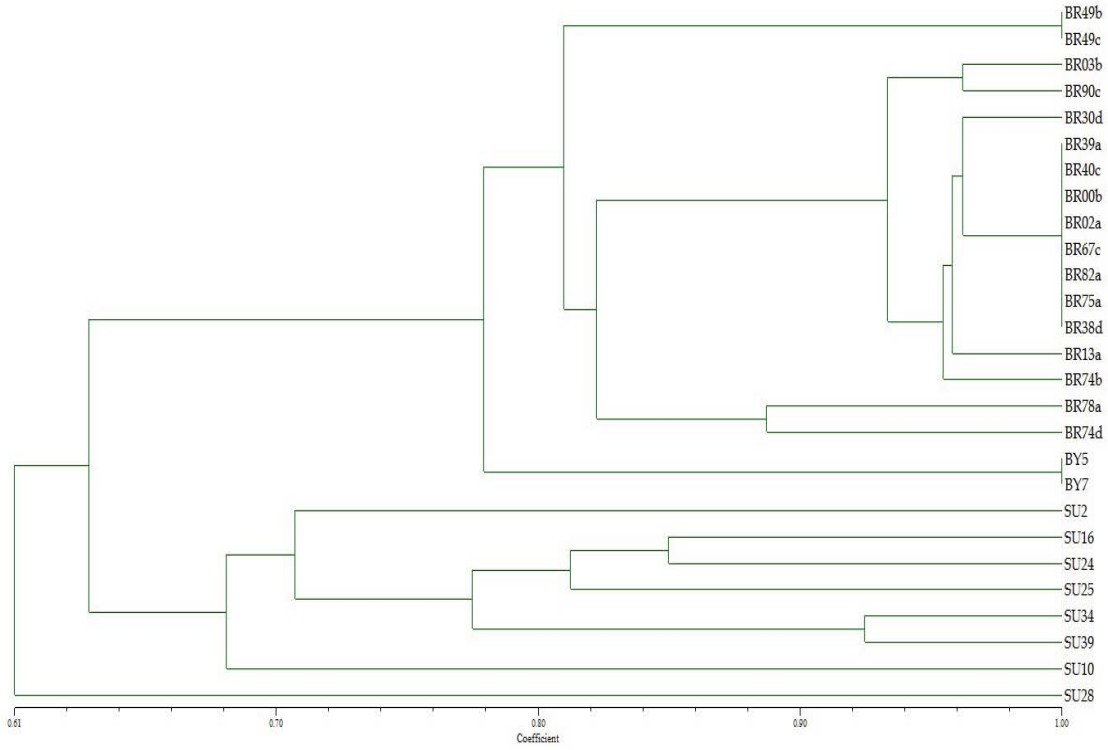

Figure 2. AFLP dendrogram analysis pattern showing relationship between 27 *S. aureus* isolates from dairy cows in Baturraden (BR), Yogyakarta (SU), Solo (SU) and Boyolali (BY) regions.

Clonal analysis of AFLP-based patterns of *S. aureus* originating from cows in Baturraden showed that there were similarities in some isolates, with values of the similarity coefficient of 100% (BR39a, BR40c, BR00b, BR02a, BR67c, BR82a, BR75a and BR38d). The results showed a close relationship between the *S. aureus* isolates originating from one location (cluster). *S. aureus* originating from Boyolali (BY5 and BY7), Solo and Yogyakarta showed different coefficients similarity, indicating the different origins of the isolates. Salasia *et al*. (2011) indicated that the clonal relationships between isolates of *S. aureus* are typically based on the origin of the isolates. Based on genotypic patterns of *S. aureus* strains, BR49b and BR49c are genotypically different from the other isolates. It is interesting that these *S. aureus* strains had 100% coefficient similarity, the same *coa* gene size (600bp), and the same *spa*-X region gene size (100bp). Boerema *et al*. (2006) and Salasia *et al*. (2011) suggested that the variations in the characteristics of genotypic patterns of *S. aureus* are influenced by the relationship between the isolates, and that clustering isolates may be partly derived from a single progenitor (ancestor).

Results obtained in this study provide information and additional knowledge about *S. aureus* infection in Baturraden cows, including the number of virulence determinant genes that may play a role in pathogenicity. We also present clonal relationship patterns among Baturraden isolates that could be important in trying to address the problems there.

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