Comparative studies on pheno- and genotypic properties of *Staphylococcus aureus* isolated from bovine subclinical mastitis in central Java in Indonesia and Hesse in Germany

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In the present study, 35 *Staphylococcus* strains isolated from milk samples of 16 cows from eight farms of three different geographic locations in Central Java, Indonesia, and from milk samples of 19 cows from 19 farms of different geographic locations in Hesse, Germany, were compared pheno- and genotypically. On the basis of cultural and biochemical properties as well as by amplification of the 23S rRNA specific to *Staphylococcus aureus*, all isolates could be identified as *S. aureus*. In addition, all *S. aureus* isolates harboured the genes *clfA* and *coa* encoding staphylococcal clumping factor and coagulase, and the gene segments encoding the immunoglobulin G binding region and the X-region of protein A gene *spa*. By PCR amplification, the genes *seb*, *seg*, *seh*, and *sei* was observed for the *S. aureus* cultures isolated in Central Java, Indonesia and the genes *sec*, *sed*, *seg*, *seh*, *sei*, *sej* and *tst* for the *S. aureus* cultures isolated in Hesse, Germany. None of the *S. aureus* of both origins harboured the genes *sea*, *see*, *eta* and *etb*. All isolates were additionally positive for the genes *nuc*, *fnbA*, *hla*, and *set1*. The gene *hlb* was found for 6 cultures from Central Java, Indonesia and 16 cultures from Hesse, Germany. However, the gene *fnbB* and the gene segments *cnaA* and *cnaB* were not present among the strains isolated in Central Java, Indonesia and rare among the strains isolated in Hesse, Germany. It was of interest that most of the *S. aureus* isolated in Central Java, Indonesia harboured the gene *cap5* and most of the strains isolated in Hesse, Germany the gene *cap8*. The phenotypic and genotypic results of the present study might help to understand the distribution of prevalent *S. aureus* clones among bovine mastitis isolates of both countries and might help to control *S. aureus* infections in dairy herds.

**Key words:** *Staphylococcus aureus*, phenotyping, genotyping, Indonesia, Germany

**Introduction**

*Staphylococcus aureus* is recognized worldwide as a major pathogen causing subclinical intramammary infections in dairy cows. The main reservoir of *S. aureus* seems to be the infected quarter, and the transmission between cows usually occurs during milking [6]. A better knowledge on the distribution of *S. aureus* in dairy herds might help to formulate strategies to reduce the spread of infection. The work of Fitzgerald *et al.* [9], Annemüller *et al.* [2], Stephan *et al.* [30] and Akineden *et al.* [1] revealed that only a few specialized clones were responsible for most of the cases of bovine mastitis in a single farm and that some of these *S. aureus* clones might have a broad geographic distribution.

*Staphylococcus aureus* produces a variety of exoproteins that contribute to the ability of this organism to cause disease in the mammalian host. These exotoxins include haemolysins, various enzymes and a family of related pyrogenic toxins, namely staphylococcal enterotoxins, toxic shock syndrome toxin, and exfoliative toxins [7]. Recently, a novel gene cluster encoding staphylococcal exotoxin-like proteins had been described [35]. Toxins related to staphylococcal pyrogenic toxins are produced by *Streptococcus pyogenes* [22]. Some of these staphylococcal toxins, also including newly described enterotoxin genes, had been described for *S. aureus* isolated from bovine mastitis [1,24].

However, at present little is known about the occurrence of these toxins among *S. aureus* isolates from Indonesia and...
about the possible distribution of single *S. aureus* clones as causative agents of bovine mastitis in various farms of one region in Indonesia. The present study was designed to comparatively investigate phenotypically and genotypically *S. aureus* isolated from milk samples of cows with subclinical mastitis in Central Java in Indonesia and Hesse in Germany.

**Materials and Methods**

**Bacterial isolates**

Thirty five isolates were obtained from milk samples of 16 cows from eight farms of three different geographic locations in Central Java, Indonesia, and from milk samples of 19 cows from 19 farms of different geographic locations in Hesse, Germany. The identification of the bacteria was performed by a tube coagulase test (Bactident-Coagulase, Merck, Germany), typical growth on Baird-Parker agar (Oxoid, Germany), and by detection of clumping factor with rabbit plasma on microscope slides [6]. The production of hemolysins of the isolates was determined by cultivation of the bacteria on sheep blood agar plates and in parallel by the interference of the hemolysins with the β-toxin of a *S. aureus* reference strain as described by Skalka et al. [28].

The production of pigment of the isolates was performed by cultivation of the bacteria on nitrocellulose membranes [20].

A molecular identification was conducted for the detection of the *S. aureus* 23S rRNA gene by using species-specific primers. The oligonucleotide primers, described by Straub et al. [31] are shown in Table 1. The reaction mixture (30 µl) contained 1 µl primer 1 (10 pmol), 1 µl primer 2 (10 pmol), 0.6 µl dNTP (10 mM; MBI Fermentas, St. Leon Rot, Germany), 3.0 µl 10X thermophilic buffer (Promega/Boehringer, Germany), 1.8 µl MgCl₂ (25 mM; Promega/Boehringer) and 0.1 µl Taq DNA polymerase (5 U/µl; Promega/Boehringer, Germany) and 20.0 µl distilled water. Finally, 2.5 µl DNA preparation was added to each 0.2 ml reaction tube. The DNA of the isolates was prepared with the QIAamp tissue kit (Qiagen, Germany) as described by the manufacturer. After cultivation of the isolates for 24 h at 37°C on blood agar plates, 5-10 colonies of the bacteria were suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA (pH 8)) containing 5 µl lysostaphin (1.8 U/µl; Sigma). After 1 h incubation at 37°C, 25 µl of proteinase K (14.8 mg/ml; Sigma, USA) and 200 µl of buffer AL (containing reagents AL1 and AL2) was added. The suspension was incubated at 56°C for 2 h and at 95°C for 10 min, and after a spin for a few seconds an amount of 200 µl ethanol was added to each sample and placed to a spin column. After centrifugation for 1 min the QIAamp spin columns were placed in a clean collection tube and the samples were washed twice with 500 µl of buffer AW (Qiagen, Germany). After a second washing and a centrifugation for 3 min, the QIAamp spin columns were placed in a clean 2 ml microfuge tube and the DNA was twice eluted with 200 µl and 100 µl of buffer AE, respectively. The amplification of the genes was carried out with thermal cycler T3 (Biometra, Germany) as described by Straub et al. [31].

**Genotypic characterization**

The genetic determinants for the following virulence traits were investigated by using oligonucleotide primers derived from the published sequences: this included the genes encoding clumping factor (*clfA*) [30], coagulase (*coa*) [14], X-region [10] and IgG binding-region of protein A (*spa*) [27], staphylococcal enterotoxins (*sea*, *34*), (*seb, sec, sed*, and *see*, 16), (*seg, seh, and sei*, 15), (*sej*, 21), TSST-1 (*tst*), exfoliative toxin A (*eta*) and B (*eb*) [16], thermonuclease (*nuc*) [5], fibronectin binding protein A (*fnbA*) and fibronectin binding protein B (*fnbB*) [4], alpha-hemolysin (*hla*) and beta-hemolysin (*hlb*) [4], collagen binding protein A domain (*cnaA*) and B domain (*cnaB*) [32], capsular polysaccharide 5 (*cap5*) and 8 (*cap8*) [23], and staphylococcal exotoxin like protein 1 (*ser1*) [35]. The sequences of the oligonucleotide primers and the temperature programs are summarized in Table 1.

**Results**

According to the results of cultural and biochemical properties as well as by amplification of the 23S rRNA specific to *S. aureus*, all 35 isolates used in the present investigation were identified as *S. aureus*. All 35 cultures were positive for coagulase, growth and tellurit reaction on Baird-Parker agar and clumping factor reaction on microscope slides. Among the 16 cultures isolated in Central Java, Indonesia, 13 cultures and among the 19 cultures isolated in Hesse, Germany, 5 cultures were positive for lipase, respectively. An α-hemolysis was observed for 3 cultures from Central Java, Indonesia and 4 cultures from Hesse, Germany, and a β-hemolysis for 10 cultures from Hesse. An αβ-hemolysis could be detected for 5 cultures from Central Java and 2 cultures from Hesse, a δ-hemolysis for 1 culture from Hesse. Eight cultures from Central Java and 2 cultures from Hesse were non-hemolytic. Cultivation of the bacteria on nitrocellulose membranes revealed that 4 cultures from Central Java and 11 cultures from Hesse produced an orange pigment, 2 cultures from both origins were yellow pigmented and 10 cultures from Central Java and 6 cultures from Hesse had a pale yellow pigment.

Amplification of the clumping factor gene *clfA* resulted in a single amplicon with a size of approximately 1000 bp from all 35 *S. aureus*, indicating no size polymorphisms of this gene. Amplification of *coa* gene yielded two different PCR products of 600 and 850 bp for 4 and 12 of the *S. aureus* isolated in Central Java, Indonesia. Five different PCR products with sizes of 510, 600, 680, 740 and 850 bp were found for 1, 10, 2, 1 and 5 of the *S. aureus* isolated in
Table 1. Primers for amplification of the gene encoding staphylococcal 23S rRNA and various other staphylococcal genes

| Gene designated | 5' primer sequence (5'-3') | 3' primer sequence (5'-3') | Size of amplified products (bp) |
|-----------------|---------------------------|---------------------------|-------------------------------|
| 23s rRNA        | ACG GAG TTA CAA AGG AGC AC| AGC TCA GCC TTA AGC AGT AC | 1250                          |
| clfA            | GGC TTC AGT GCT TGT AGG  | TGG TCA GGG GTA CAA TAA GC | size polymorphisms            |
| coa             | ATA GAG ATG CTG GTA CAG G| GCT TCC GAT TGT TCG AGT C  | size polymorphisms            |
| spa (IgG-binding region) | CAC CTG CTG CAA ATG CTG CG | GGC TTA TTG TTA TTG TTA | size polymorphisms |
| spa (X-region)  | CAA GCA CCA AAA GAG GAA  | CAC GAG TGT TAA CAA CAT   | size polymorphisms            |
| sea             | AAA GTC CCG ATC ATA TTA TGG CTA | GTA ATT AAC CGA AGG TTC TGT AGA | 216 |
| seb             | TCG CAT CAA ACT GAC AAA CG | GCA GGT ACT CTA TAA GTG CC | 478 |
| sec             | GAC ATA AAA AGG AGT ATT TT | AAA TCG GAT TAA CAT TAT C  | 257 |
| sed             | CTA GTT TGG TAA TAT CTC CT | TAA TGA TAT CAT TTA TAG GG | 317 |
| see             | TAG ATA AGG TTA AAA CAA GC | TAA CTT ACC GTG ACC CTT TC | 170 |
| seg             | AAT TAT GTG AAT GCT CAA CCC GAT C | AAA CTT ATA TGG AAC AAA AGG TAG TGG | 642 |
| seh             | CAA TCA CAT CAT ATG CGA AAG CAG | CAT CTA CCC AAA CAT TAG CAC C | 375 |
| sei             | CTC AAG GTG AAT TTA TGG TTA TGG | AAA AAA TTT ACA GGC AGT CCA TCT C | 576 |
| sej             | CAT GAC AAC TGT TGG TCC GTT AG | CTT AAA TTT ACC AAT AAA GGT AC | 142 |
| tst             | ATG GCA GCA TCA GCT TGA TA | TTA CCC AAT ACC ACC GTT TT | 350 |
| eta             | CTA GTG CAT TTA TTA TTA TGA | TGC ATT GAC ACC ATA GTA CT | 120 |
| etb             | ACG GCT ATG TTA ACA TTA TT | TCC ACC ATG AAT ATA CCT AA | 201 |
| nuc             | GGC ATT GAT GTT GAT AGC GTT | ACG CAA GCC TGT AGC AAC TAA AGC | 279 |
| fnbA            | GGC GAG ATC AAA GAC AA   | CCA CCT AAT GTA GTC TGT G | 1279 |
| fnbB            | GGA GAA GGA ATT AAG GGC | GCC GTC GCC TGG AGC GT | 812 |
| hla             | GGT TTA GCC TGG CTT TC | CAT CAC GAA CTC GTT GC | 534 |
| hlb             | GCC AAA GCC GAA TCT AAG | GGC ATA ATG ATC CCA TGG C | 833 |
| cna (A domain)  | ATA TGA ATT CGA GTA TTA GGA GGG GT T | TTT GGA TCC CTT TTT CAG TAT TAG TAA CCA | 1200 |
| cna (B domain)  | AGT GGT TAC TAA TAC TG | CAG GAT AGA TTG GGT TA | 1738 |
| cap5            | ATG AGC ATG AGG ATA GCG | CTC GGA TAA CAC CTG TTT C | 880 |
| cap8            | ATG AGC ATG AGG ATA GCG | CAC CTA ACA TAA GGC AAG | 1147 |
| setl            | GGT TAA TTC ATA GGC CAG TAT C | CAA CGT TTA ATG GGT AAG CTG C | 879 |
from Hesse, Germany, respectively. PCR amplification of the gene segment encoding the IgG-binding region of protein A revealed a size of 900 bp from 32 of the isolates investigated from Central Java, Indonesia and Hesse, Germany. However, the protein A gene of three cultures from Hesse, Germany revealed an amplicon size of 780 bp. Amplification of the X-region of spa gene of the S. aureus isolated from Central Java, Indonesia showed two different sized amplicons of 270 and 320 bp for 6 and 10 isolates, respectively. On the other hand, 9 different sized amplicons of 100, 150, 200, 230, 240, 250, 270, 290 and 340 bp were observed for 8, 1, 1, 1, 2, 1, 1, 2 and 2 S. aureus isolated in Hesse, Germany, respectively. Some phenotypic and genotypic properties of the 35 S. aureus isolates are summarized in Table 2.

Among the 16 S. aureus cultures isolated in Central Java, Indonesia 1 culture harboured the genes seb and seh, and 3 cultures the genes seg and sei. Among the S. aureus isolated in Hesse, Germany the gene sec was observed for 11 cultures, seh for 3 cultures, sed and sej for 3 cultures, seg and sei for 12 cultures, respectively. All 11 isolates containing sec were simultaneously positive for tst. None of the S. aureus isolate in Central Java, Indonesia and Hesse, Germany harboured the genes encoding sea, see, eta and eth. All isolates were additionally positive for the genes mnc, fnbA, hla, and set1. The gene fnbB was observed for 1 culture from Hesse, Germany, the gene hlb for 6 cultures from Central Java, Indonesia and 15 cultures from Hesse, Germany, the gene segments cnaA and cnaB for 2 cultures from Germany, the gene cap5 for 15 cultures from Central Java and 7 cultures from Hesse, Germany, and the gene cap8 for 1 culture from Central Java, Indonesia and 12 cultures from Hesse, Germany, respectively. Amplicons specific to typical hla, hlb, cap6 and cap8 are shown in Fig. 1 and Fig. 2. The distribution of the various genes among the S. aureus cultures of both origins are summarized in Table 3.

**Discussion**

According to pheno- and genotypic properties all 35 isolates investigated in the present study could be identified as S. aureus. The molecular identification and characterization were performed by PCR amplification of the genes encoding the 23S rRNA, clumping factor, coagulase, and the gene segments encoding the immunoglobulin G binding region and the X- region of protein A. A comparable PCR-based system for identification of S. aureus isolated from various origins had already been used in previous paper [1,2,30,31].

Investigating the S. aureus isolates for toxin genes revealed that, besides seb, the newly described enterotoxin genes seg, seh and sei could be observed for some S. aureus isolated in Central Java, Indonesia. However, the toxin genes sec, seg, sei and tst seemed to be the predominant toxin genes of S. aureus isolated in Hesse, Germany. The combined occurrence of the toxin genes seg and sei, sed and sej, sec and tst of S. aureus, observed in the present study had also been described by Zhang et al. [36], Jarraud et al. [15], Stephan et al. [30] and Akineden et al. [1], and could be explained by a combined location of these genes on pathogenicity islands [3,18] and on a plasmid [36]. The importance of toxin formation of S. aureus isolated from bovine mastitis for udder pathogenesis remains unclear.
Table 2. Pheno- and genotypic characteristics of *S. aureus* isolates from Central Java, Indonesia and Hesse, Germany

| Origin       | Hemolysis | Pigment | clfA gene (bp) | coa gene (bp) | IgG binding region (bp) | X-region (bp) | spa gene |
|--------------|-----------|---------|----------------|---------------|-------------------------|---------------|---------|
|              | α | β | α/β | δ | non | o | y | py | 1000 | 510 | 600 | 680 | 740 | 850 | 780 | 900 | 100 | 150 | 200 | 230 | 240 | 250 | 270 | 290 | 320 | 340 |
| Central Java (n=16) | 3* | - | 5 | - | 8 | 4 | 2 | 10 | 16 | - | 4 | - | - | 12 | - | 16 | - | - | - | - | - | - | 6 | 10 | - |
| Hesse (n=19)    | 4 | 10 | 2 | 1 | 2 | 11 | 2 | 6 | 19 | 1 | 10 | 2 | 1 | 5 | 3 | 16 | 8 | 1 | 1 | 1 | 2 | 1 | 1 | 2 | - | 2 |

n = number of cultures
* = number of cultures with the respective property
o = orange; y = yellow; py = pale yellow

Table 3. Distribution of potential virulence genes among *S. aureus* isolated in Central Java, Indonesia and Hesse, Germany as determined by PCR analysis

| Source       | a | b | c | d | e | g | h | i | j | tst | eta | eth | nuc | fnbA | fnbB | hla | hlb | cnaA | cnaB | cap5 | cap8 | set1 |
|--------------|---|---|---|---|---|---|---|---|---|-----|-----|-----|-----|------|------|-----|-----|------|------|------|------|------|
| Central Java (n=16) | - | 1* | - | - | - | 3 | 1 | 3 | - | - | - | - | 16 | 16 | - | 16 | 6 | - | - | 15 | 1 | 16 |
| Hesse (n=19)     | - | - | - | 11 | 3 | 12 | 3 | 12 | 3 | 11 | - | - | 19 | 19 | 1 | 19 | 15 | 2 | 2 | 7 | 12 | 19 |

n = number of cultures
* = number of cultures with the respective property
According to Ferens et al. [8], the superantigenic toxins seem to induce an immunosuppression in dairy animals. None of the strains isolated from Central Java, Indonesia and Hesse, Germany harboured the genes see, see, eta and etb. Hayakawa et al. [13] reported that the production of exfoliative toxins among S. aureus isolates from cattle with bovine mastitis seems to be rare.

A PCR investigation of additional genetic determinants revealed that the genes nuc, fnbA, hla, and set1 were found in all strains investigated, suggesting an important role of these elements for pathogenicity in bovine mastitis. However, fnbB and the gene segments cnaA and cnaB were not present among the strains isolated from Central Java, Indonesia and rare among strains isolated from Hesse, Germany. Jonsson et al. [17] described that the two S. aureus fibronectin-binding proteins and their corresponding genes have a high degree of sequence similarity. The fibronectin-binding proteins of S. aureus are important virulence factors and contribute to bacterial adhesion and to invasion of the bovine mammary gland [19]. However, mutants defective in either of the two fnb-genes adhered equally well to fibronectin [11]. In the present study fnbA was detected in all isolates and fnbB only in 1 S. aureus isolated in Hesse, Germany. Booth et al. [4] observed that 89.7% of the investigated strains possessed fnbA, whereas only 20.1% harboured fnbB. The gene set1 represents a newly described toxin group which appears in numerous allelic variants [3,18,35]. At present the occurrence of these allelic variants among S. aureus from bovine mastitis is not known. The gene cna was found in 2 S. aureus isolated in Hesse, Germany. The ability of S. aureus to adhere to extracellular matrix proteins is thought to be essential for colonization and the establishment of infection. The gene cna is the only recognized gene that encodes an adhesin that especially binds collagen [25], and it is the only adhesin protein gene that is not present in all S. aureus strains [4,23,29]. However, cna seems to be of minor importance for adhesion of S. aureus from bovine mastitis. It was of interest that the S. aureus isolated from Central Java, Indonesia generally harbour the gene cap5, and that gene cap8 was frequently found among the S. aureus strains from Hesse, Germany. S. aureus might express up to 11 polysaccharide capsular types [33]. However, most strains from bovine milk could be classified to type 5 and 8 [12,26]. The extracellular polysaccharide capsule is particularly relevant to bovine mastitis, since 94 to 100% of S. aureus strains isolated from cows with mastitis are encapsulated [12].

According to the results of the present study S. aureus isolated from bovine mastitis in Central Java, Indonesia and Hesse, Germany showed only minor differences in their gene patterns indicating that the described virulence traits seem to be also of importance for S. aureus from bovine mastitis of both countries. In addition, the phenotypic and genotypic results of the present study might help to understand the distribution of prevalent S. aureus clones among bovine mastitis isolates, which can be the base to investigate and control the hitherto unknown route of S. aureus infections in Indonesian dairy herds.

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