J. Vet. Sci. (2004), **5**(2), 103–109

JOURNAL OF
Veterinary
Science

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 Staphylococcal strain 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, fnb*A, *hla*, and *set*1. The gene *hlb* was found for 6 cultures from Central Java, Indonesia and 16 cultures from Hesse, Germany. However, the gene *fnb*B and the gene segments *cna*A and *cna*B 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 *cap*5 and most of the strains isolated in Hesse, Germany the gene *cap*8. 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

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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.

S. 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 ß-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 $MgCl_2$ (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 (*etb*) [16], thermonuclease (*nuc*) [5], fibronectin binding protein A (*fnb*A) and fibronectin binding protein B (*fnb*B) [4], alpha-hemolysin (*hla*) and beta-hemolysin (*hlb*) [4], collagen binding protein A domain (*cna*A) and B domain (*cna*B) [32], capsular polysaccharide 5 (*cap*5) and 8 (*cap*8) [23], and staphylococcal exotoxin like protein 1 (*set*1) [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, a β-hemolysis for 10 cultures from Hesse. An α/β-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 *clf*A 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 ACG AC	AGC TCA GCC TTA ACG AGT AC	1250
clfA	GGC TTC AGT GCT TGT AGG	TTT TCA GGG TCA ATA TAA GC	size polymorphisms
coa	ATA GAG ATG CTG GTA CAG G	GCT TCC GAT TGT TCG ATG C	size polymorphisms
spa (IgG-binding region)	CAC CTG CTG CAA ATG CTG CG	GGC TTG TTG TTG TCT TCC TC	size polymorphisms
spa (X-region)	CAA GCA CCA AAA GAG GAA	CAC CAG GTT TAA CGA CAT	size polymorphisms
sea	AAA GTC CCG ATC AAT 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 GCT AGG AAT TT	AAA TCG GAT TAA CAT TAT CC	257
sed	CTA GTT TGG TAA TAT CTC CT	TAA TGC TAT ATC TTA TAG GG	317
see	TAG ATA AGG TTA AAA CAA GC	TAA CTT ACC GTG GAC CCT TC	170
seg	AAT TAT GTG AAT GCT CAA CCC GAT C	AAA CTT ATA TGG AAC AAA AGG TAC TAG TTC	642
seh	CAA TCA CAT CAT ATG CGA AAG CAG	CAT CTA CCC AAA CAT TAG CAC C	375
sei	CTC AAG GTG ATA TTG GTG TAG G	AAA AAA CTT ACA GGC AGT CCA TCT C	576
sej	CAT CAG AAC TGT TGT TCC GCT AG	CTG AAT TTT ACC ATC AAA GGT AC	142
tst	ATG GCA GCA TCA GCT TGA TA	TTT CCA ATA ACC ACC CGT TT	350
eta	CTA GTG CAT TTG TTA TTC AA	TGC ATT GAC ACC ATA GTA CT	120
etb	ACG GCT ATA TAC ATT CAA TT	TCC ATC GAT AAT ATA CCT AA	201
nuc	GCG ATT GAT GGT GAT ACG GTT	ACG CAA GCC TTG ACG AAC TAA AGC	279
fnbA	GCG GAG ATC AAA GAC AA	CCA TCT ATA GCT GTG TGG	1279
fnbB	GGA GAA GGA ATT AAG GCG	GCC GTC GCC TTG AGC GT	812
hla	GGT TTA GCC TGG CCT TC	CAT CAC GAA CTC GTT CG	534
hlb	GCC AAA GCC GAA TCT AAG	GCG ATA TAC ATC CCA TGG C	833
cna (A domain)	ATA TGA ATT CGA GTA TAA 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 GTT TA	1738
*cap*5	ATG ACG ATG AGG ATA GCG	CTC GGA TAA CAC CTG TTG C	880
*cap*8	ATG ACG ATG AGG ATA GCG	CAC CTA ACA TAA GGC AAG	1147
*set*1	GGT TAA TTC ATA GCG CAG TAT C	CAA CGT TTC ATC GTT AAG CTG C	879

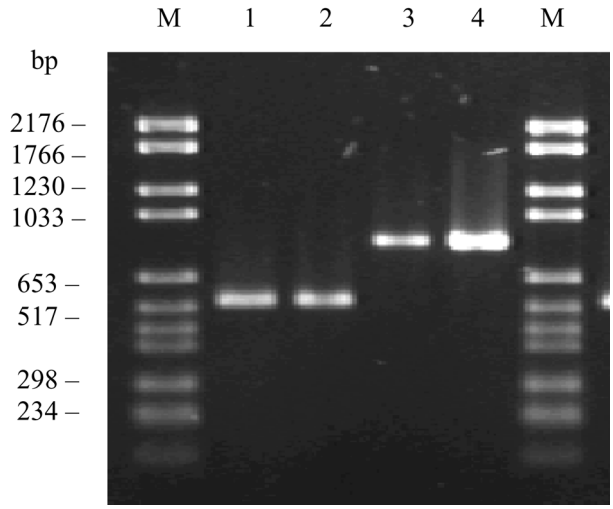

Fig. 1. Typical amplicons of the genes encoding staphylococcal α-toxin (*hla*) and β-toxin (*hlb*) of *S. aureus* with size of 534 bp (*hla*, lanes 1-2) and 833 bp (*hlb*, lanes 3-4). M, DNA molecular weight marker VI (Roche, Mannheim, Germany).

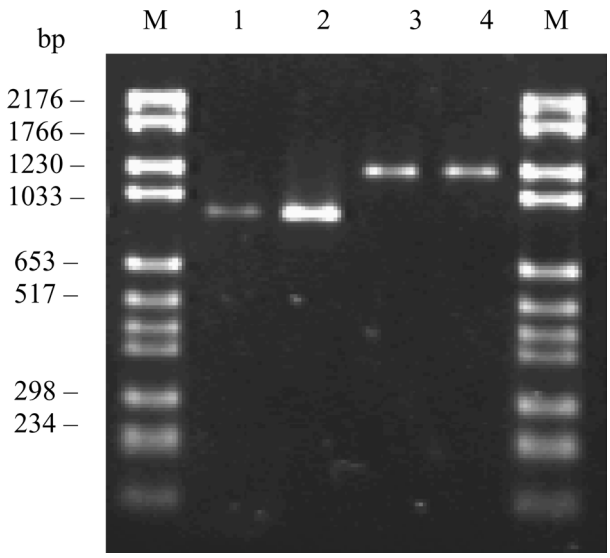

Fig. 2. Amplicons of the genes encoding staphylococcal capsular polysaccharide 5 (*cap*5) and 8 (*cap*8) of *S. aureus* with size of 880 bp (*cap*5, lanes 1-2) and 1147 bp (*cap*8, lanes 3-4). M, DNA molecular weight marker VI (Roche, Mannheim, Germany).

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 *etb.* All isolates were additionally positive for the genes *nuc*, *fnb*A, *hla*, and *set*1. The gene *fnb*B 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 *cna*A and *cna*B for 2 cultures from Germany, the gene *cap*5 for 15 cultures from Central Java and 7 cultures from Hesse, Germany, and the gene *cap*8 for 1 culture from Central Java, Indonesia and 12 cultures from Hesse, Germany, respectively. Amplicons specific totypical *hla*, *hlb*, *cap*6 and *cap*8 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			*clf*A gene (bp)		*coa* gene (bp)				*spa* gene										
															IgG binding region (bp)		X-region (bp)								
	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	se-									tst	eta	etb	nuc	*fnb*A	*fnb*B	hla	hlb	*cna*A	*cna*B	*cap*5	*cap*8	*set*1
	a	b	c	d	e	g	h	i	j													
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 immunosuppresion in dairy animals. None of strains isolated from Central Java, Indonesia and Hesse, Germany harboured the genes *sea*, *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*, *fnb*A, *hla*, and *set*1 were found in all strains investigated, suggesting an important role of these elements for pathogenicity in bovine mastitis. However, *fnb*B and the gene segments *cna*A and *cna*B 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 *fnb*A was detected in all isolates and *fnb*B only in 1 *S. aureus* isolated in Hesse, Germany. Booth *et al*. [4] observed that 89.7% of the investigated strains possessed *fnb*A, whereas only 20.1% harboured *fnb*B. The gene *set*1 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 specially 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 harboured the gene *cap*5, and that gene *cap*8 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.

Acknowledgments

This work was supported by Alexander von Humboldt Foundation, Germany. The authors wish to thank Dr. Jörg Alber for analysis of *set*-gene and the design of *set*1-primers.

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